Volume Regulation by Articular Chondrocytes

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Declaration

The work presented in this thesis was performed in the Membrane Biology Group (MBG), School of Biomedical and Clinical Laboratory Science, University of Edinburgh. The composition of this thesis is my own work unless stated otherwise.

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Dedication

I would like to dedicate this thesis to my Mum, who I only knew for a short time but will remember forever. Thank you.

-x-

Abstract

Chondrocytes, the only resident cell type in articular cartilage are entirely responsible for the synthesis and breakdown of the extracellular matrix in response to the physico-chemical environment (Stockwell, 1991). A failure of this regulation leads to the onset of osteoarthritis where one of the first macroscopic events that occurrs is cartilage overhydration. Previous work has shown that changes in extracellular osmolality affect matrix synthesis (Urban, 1994; Hopewell & Urban, 2003) although it is currently unknown how this influences chondrocyte cell-volume. Here, chondrocyte volume-regulation was studied in bovine and human chondrocytes isolated from articular cartilage.

Load-bearing, articular chondrocytes were excised aseptically and the chondrocytes isolated using a standard technique into 380mOsm.kg H_2O^{-1} DMEM (Hall *et al.*, 1996b). For measurements of cell volume and intracellular calcium ($[Ca^{2+}]_i$), chondrocytes were incubated with fura-2 AM (5μM; 30mins;37°C) and experiments performed as previously described (Kerrigan & Hall, 2000). A 43% hyper and hypo-osmotic challenge was applied (by perfusion) to cause cell shrinkage and swelling respectively. Data were expressed as mean ± s.e.m. with the number of joints (n) and cells (N).

In response to a decrease in extracellular osmolality some chondrocytes were able to volume-regulate (termed Regulatory Volume Decrease; RVD) where RVD was found to be independent of an intact actin cytoskeleton, not dependent upon stretch-activated calcium channels, attenuated by the removal of extracellular calcium and inhibited by

REV 5901 (non-specific inhibitor of chondrocyte volume-regulation; (Bush & Hall, 2001b). A decrease in osmolality resulted in a rise in $[Ca^{2+}]_i$ in some cells that did not appear to correlate with the capacity for RVD in some cells. Conversely, in response to a hyper-osmotic challenge there was an absence of any volume-regulation (termed Regulatory Volume Increase; RVI) despite the expression of the Na-K-2Cl co-transporter. The 'post RVD-RVI protocol' did not significantly increase the number of cells showing RVI despite robust RVD. As with a decrease in osmolality, there was a corresponding rise in $[Ca^{2+}]_i$ that did not correlate with volume-regulation.

When comparing the capacity for RVD between chondrocytes isolated from macroscopically 'degenerate' and 'non-degenerate' human articular cartilage no differences were found. RVD was not dependent upon stretch-activated calcium channels although unlike bovine articular chondrocytes, was not inhibited by REV 5901.

These data show that some chondrocytes have the capacity for volume regulation and this appears to be calcium independent in some cells whereas in other cells RVD was dependent upon the intracellular calcium rise. It would appear that the cellular mechanisms for sensing and/or responding to changes in cell-volume may be different to that for membrane-stretch and that the differential state of the cell may be important. The mechanism for RVD in human articular chondrocytes appears to differ to that in avian and bovine chondrocytes although is not attenuated in chondrocytes isolated from 'degenerate' cartilage when compared to 'non-degenerate' controls.

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Abbreviations

2D	2-Dimensions
3D	3-Dimensions
Å	Angstrom
AM ester	Acetoxymethyl ester
atms	Atmospheres
ATP	adenosine triphosphate
AtT20	Mounse Corticotrophic cell line
BACs	Bovine Articular Chondrocytes
BK	Large conductance Ca^{2+} activated K^{+} channels
Br^-	Bromine
^{14}C	Radioactive Carbon (fourteen)
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular calcium
$[Ca^{2+}]_o$	Extracellular calcium
$CaCl_2$	Calcium Chloride
CaCC	calcium sensitive chloride channels
cAMP	Cyclic Adenosine Mono Phosphate
CCC	Cation–Chloride Cotransporter family
cGMP	Cyclic Guanosine Monophosphate
Cl^-	Chlorine
$[Cl^-]_i$	Intracellular Chloride
$[Cl^-]_o$	Extracellular Chloride
Cl^-/HCO_3^-	Chloride Bicarbonate Exchange
CLSM	Confocal Laser Scanning Microscopy
CMFDA	5-chloromethylfluorescein diacetate
CO_2	Carbon Dioxide
Da	Dalton
DAG	Diacylglycerol
ddH_2O	Double Distilled Water
DIDS	4,4'-diisothiocyanitosilbene-2,2'- disulphic acid
NDGA	Nordihydroguaiaretic Acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Di-Methyl Sulfoxide
DZ	Deep Zone of Cartilage
EAT cells	Ehrlich Ascites Tumour cells
ECM	Extracellular matrix
EDTA	Ethylene Diamine Tetra-acetic Acid
EGTA	Ethylene Glycol Bis(2-aminoethyl ether)tetraacetic Acid Ethylenebis(oxyethylenenitrile)tetraacetic Acid
EM_λ	Emission Wavelength
ER	Endoplasmic Reticulum
ERK	Extracellular signal-Regulated protein Kinase
EX_λ	Excitation Wavelength
F^-	Fluoride

F-actin	Polymerised actin
FAC	Focal adhesion Complex
FCS	Foetal Calf Serum
FCD	Fixed Charge Density
FITC	Fluoresceine Iso-Thio Cyanate
Fu.min^{-1}	Fluorescence Units per minute x10^3
Fura-2	1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′N′-tetraacetic Acid
G-actin	Monomeric actin
GABA	Gamma Aminobutyric Acid
GAG(s)	Glycoaminoglycan(s)
Gd^{3+}	Gadolinium
^3H	Radioactive Hydrogen
H^+	Hydrogen
$[H^+]$	Hydrogen ion concentration
HCO_3^-	Bicarbonate
HEC cells	Human Endothelium Cells
HEPES	N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid].
Hrs	Hours
Hz	Hertz
IC_{50}	inhibitory concentration 50%
IL	Interleukin
IP_3	Inositol (1,4,5)-Tri-Phosphate
K^+	Potassium
$[K^+]_i$	Intracellular potassium
$[K^+]_o$	Extracellular potassium
K_{ATP}	ATP-sensitive potassium channel
KCC	K^+ Cl^- cotransporter
KCl	Potassium Chloride
kg/mm^2	Kilogram per mm squared
Kv	Voltage gated K^+ channel
Mb	Megabyte
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium Chloride
ML-7	Inhibitor of MLCK
MLCK	myosin light chain kinase
mmol/L	Millimole per litre
Mn^{2+}	Manganese
MMP	Matrix Metalloprotease
MN/m^2	Mega-Newton's per meter squared
mOsm.kg H_2O^{-1}	Milliosmolyes per kg of water
MPa	Mega-Pascal
mRNA	Messenger RNA (ribonucleic acid)
MZ	Mid Zone of cartilage
Na^+	Sodium
$[Na^+]_o$	Extracellular sodium

NaCl	Sodium Chloride
Na^+/HCO_3^- cotransporter	Sodium bicarbonate cotransporter
NDGA	Nordihydroguaiaretic acid
NEM	N-ethylamleimide
NHE	Na^+/H^+ exchanger
NHS	National Health Service
NKCC	Na^+-K^+-$2Cl^-$ Co-transporter
NPPB	5-Nitro-2-(3-phenylpropylamino)- benzoic acid
NO	Nitric Oxide
NO_3^-	Nitrate
OA	Osteoarthritis
PBS	Phosphate Buffered Saline
PC	Personal Computer
PG(s)	Proteoglycan(s)
pI_{Cln}	Outwardly rectifying anion channel
PKA	Protein Kinase A
pS	Pico seconds
PTI	Photon Technology International
RA	Rheumatoid Arthritis
^{86}Rb	Radioactive ^{86}Rubidium
RVD	Regulatory Volume Decrease
REV 5901	α-Pentyl-3-(2-quinolinylmethoxy)benzyl alcohol
ROI	Region Of Interest
RVI	Regulatory Volume Increase
SAC	Stretch Activated Channel
SCN^-	Thiocyanate
S.E.M	Standard Error of the Mean
$^{35}SO_4$	Radioactive sulphate
SAPK2	Stress-activated Protein Kinase 2
SITS	4-acetamido-4'- isothiocyanostilbenezene-2, 2-disulfonic acid
SK	Small Conductance Potassium Channel
SZ	Superficial Zone of cartilage
$t_{½}$	Time for 50% RVD
TEA	Tetraethylammonium
TNF	Tissue Necrosis Factor
TRITC	Tetramethyl Rhodamine Iso-Thio Cyanate
TRPV	Transient Potential Receptor Cation Channel
UNIX	The acronym for 'Multiplexed Information and Computing SE' was Multix. Since the opposite of Multi is Uni, Unix was born.
V_{final}	Final change in volume at end of experimental period
V_{max}	Maximal change in cell volume
V_{rest}	Volume recorded in iso-osmotic saline
VACC	Voltage Activated Calcium Channel

VRAC	Volume Regulated Anion Channel
VSAC	Volume Sensitive Anion Channel
VSOAC	Volume Sensitive Organic Osmolyte and Anion Channel
v/v	Volume per volume
w/v	Weight per volume

Introduction

1.0.0 The Function of Articular Cartilage

Articular cartilage is an avascular, aneuronal tissue found on the opposing surfaces of synovial joints (Stockwell, 1979; Stockwell, 1991; Buckwalter & Mankin, 1997a). In conjunction with synovial fluid, it provides a smooth, low-friction surface suitable for joint movement, thus protecting the underlying bone from compressive and shearing forces generated by joint articulation (Stockwell, 1991). As articular cartilage is slightly malleable (a Young's modulus of 8-15 MN/m^2 compared to that of 9 MN/m^2 for bone; Harkness, 1968) it also provides a degree of protection from static compressive load by dissipating the weight over a larger surface area (Weightman & Kempson, 1979b; Stockwell, 1991). Cartilage is comprised of an extracellular matrix (ECM) synthesised by the only resident cell type, chondrocytes, where these cells represent only ~5% of the tissue volume (human femoral head; (Stockwell, 1979). Chondrocytes are able to 'sense' and respond to changes in the physico-chemical environment (as a result of mechanical loading), thus producing a suitable ECM and maintaining the functional integrity of the cartilage (Grushko *et al.*, 1989; Urban, 1994).

1.1.0 The Organisation of Articular Cartilage

1.1.1 Collagen

Cartilage comprises principally of water (65-80%), collagen (15-25%) and proteoglycan (3-10%) with small amount of noncollagenous and nonproteoglycan protein (Maroudas, 1980). The principal collagen in adult articular cartilage is type II (accounting for 90%

of all cartilage collagen; (Sandell, 1995) with small amounts of type IX (although as it has a chondroitin sulphate chain it is often regarded as a proteoglycan) and type XI also present (Stockwell, 1979; Bruckner & Vanderrest, 1994; Eyre, 2002). Collagen type II is a homotrimeric protein with a characteristic Gly-X-Y triplet repeat forming (by triplet) right-handed helical fibrils. Conversely, collagen type IX is a highly glycosylated, non-fibular heterotrimer that binds to the surface fibrils of type II collagen, cross-linking the cartilage matrix and thus forming a collagen network (Eyre, 1987; Shimokomaki *et al.*, 1990; Bruckner & Vanderrest, 1994; Eyre, 2002).

The organisation of the collagens within articular cartilage was first described by (Benninghoff, 1925) and then modified by Broom in the 1980s (Broom, 1984). Based on the morphology and volume of the chondrocytes (and the organisation and arrangement of collagen fibrils that they synthesise), cartilage is subdivided into 3 principle zones termed: superficial (SZ), mid (MZ) and deep (DZ; (Buckwalter, 1992; Buckwalter & Mankin, 1997a). In the SZ, the collagen fibrils are tightly packed, run parallel to the articular surface and have an average diameter of 320 ± 50 Å (Weiss *et al.*, 1968). This orientation, in conjunction with the high tensile strength of collagen II (15-30 kg/mm^2; (Harkness, 1968) helps to confer resistance to the tensile forces generated during joint articulation.

In the MZ, the collagen fibrils appear less organised, vary in diameter from 300 to 600 Å with proteoglycan (PG) between each fibre (Weiss *et al.*, 1968). Unlike the collagens of the superficial zone, the fibres are not arranged in bundles and do not follow the line of the articular surface (Stockwell, 1991); Buckwalter & Mankin, 1997a; (Hall, 1998).

Instead, the overall organisation generally resembles a 'Basket weave' with the fibres radial to the articular surface interweaving with each other and possibely linked together by collagen IX (Weiss *et al.*, 1968; Eyre, 1987). The organisation of the MZ collagen fibrils immobilises the PG within the tissue, restricts the swelling of the PG to ~20% of their hydrated volume (Hascall *et al.*, 1981) and consequently the MZ is kept under tension. This tension confers a resistance to the static compressive forces generated during joint articulation. In response to load, the degree by which cartilage deforms is dependent upon the scale of the force where conversely, the extent of fluid loss is dependent upon duration (Urban, 1994). The deformation in response to load therefore protects the underlying bone from the cyclic impact generated during joint articulation.

Collagens of the DZ are organised differently when compared to the other two zones. The fibres have the largest variation in diameter with fibrils measuring from 400 to 800 Å and are anchored to the calcified layer of cartilage (found just below the DZ) but do not cross the osteochondral junction (Weiss *et al.*, 1968; Skolofff, 1973; Eyre, 1987; Stockwell, 1991; Eyre & Wu, 1995; Eyre, 2002). As described previously for the MZ, the DZ mainly experiences compressive forces and therefore the PG within the collagen network confers a resistance.

1.1.2 Proteoglycan

Proteoglycans (PGs) are the other main proteinaceous constituent of the extracellular matrix. They comprise of a core protein covalently bound to large sulphated glycoaminoglycan (GAG) chains of either chondroitin or keratan sulphate. The largest of the articular cartilage PGs is aggrecan (made of monomeric glycoaminoglycan units)

that interacts with hyaluronan (a non-sulphated GAG) and link protein to make large complexes in the order of 5 x 10^7 to 5 x 10^8 Da (Broom & Poole, 1983; Hascall, 1988; Buckwalter *et al.*, 1989; Hardingham *et al.*, 1990; Hardingham *et al.*, 1994; Redini, 2001). These large complexes are therefore trapped within the collagen network and constrained so they are not fully hydrated (Hascall, 1988). As previously mentioned, this subsequently confers resistance to the compressive forces generated during joint articulation (Urban, 1994). Clearly, changes in the PG type or concentration (as a result of changes in either catabolism or anabolism) will alter the physical properties of the ECM and therefore the integrity and mechanical resilience of the cartilage matrix (Handley *et al.*, 1985).

Unlike the PG of the mid and deep zones, in the superficial zone the amount of PG differs. When compared to the other zones there is a low concentration of PG and a high concentration of collagen and this can be related to function (Buckwalter *et al.*, 1997). The dense collagen network and the lower levels of PG help to confer the mechanical properties of the tissue and influence the permeability of the cartilage. It has been shown that the removal of the SZ, increases the permeability of the cartilage and therefore potentially weakening the matrix making the tissue or susceptible to OA.

Besides the large chondroitin or keratan sulphate rich PGs, articular cartilage is also comprised of smaller leucine-rich PGs namely fibromodulin, decorin and biglycan. Decorin and fibromodulin (structurally similar although having different numbers of dermatan sulphate chains) both interact with collagen type II and help mediate inter-fibril interactions (Rosenberg & Hunziker 2001). Conversely, biglycan (comprised of

several dermatan sulphate chains) is mainly located in the pericellular matrix (forming part of the chondron structure) and interacts with collagen type VI (Kuettner *et al.*, 1991; Roughley & Lee, 1994).

The PGs are negative charged due to carboxyl and sulphonic groups of the chondroitin and keratan sulphates (Maroudas, 1980). This in conjunction with the fact that the PGs are immobilised by the collagen network results in an unusual ionic environment when compared to other tissues (Table 1.1). There are raised levels of cations (principally Na^+, K^+ and Ca^{2+}), decreased level of anions (Cl^- and HCO_3^-) and as a result there is an influx of water (from the synovial fluid) into the tissue until an equilibrium with the extracellular matrix is reached (Maroudas, 1980; Urban, 1994). This is an example of the Donnan Equilibrium and is pivotal to understanding and the function of cartilage.

As collagen (excluding type IX) does not carry a net charge (Maroudas, 1980); (Hall, 1998), fixed charge density (FCD) analysis has been used to calculate the distribution of proteoglycan within articular cartilage (Maroudas, 1980). It has been shown that PG concentration is lowest in the SZ, increases in the MZ and upper DZ and then begins to decrease (Bayliss *et al.*, 1983; Broom & Poole, 1983; Handley *et al.*, 1985; Heinegard, 1993). This organisation is the result of the synthetic activity of the chondrocytes, dependent upon the zone they are in, and consequently the forces they perceive (Buckwalter & Mankin, 1997a). In the SZ, the collagens fibrils are arranged close together with little PG in between the fibres (Weiss *et al.*, 1968; Hall, 1998). Subsequently, the SZ is less hydrated when compared to the MZ and DZ as cartilage hydration follows FCD and in conjunction with the fact it has the lowest chondrocyte

density (17% compared to 53% and 30% of the MZ and DZ respectively) confers this zone with resistance to shearing forces (Buckwalter & Mankin, 1997a; Hall, 1998). Conversely, in the MZ and DZ there is an increase in the amount of PG (and consequently an increase in FCD) that increases the hydration of these zones and makes them more resilient to compressive forces (Stockwell, 1991). Overall, this gives rise to a complex hydration profile that is necessary for the correct function of the articular cartilage.

As well as structural proteins, cartilage also comprises of other proteins involved in the regulation of the extracellular matrix. Anchorin and Cartilage Oligomeric Protein (COMP) are proteins involved in linking the cartilage fibrils to the chondrocytes with the later is often used as a marker of OA (Buckwalter *et al.*, 1997). Fibronectin and tenascin are also involved in linking the chondrocytes to the extracellular matrix but have also been implicated in the matiance and repair of the extracellular matrix by stimulating PG synthesis (Martin & Buckwalter, 1998).

	$[Na^+]_o$ (mM)	$[K^+]_o$ (mM)	$[Ca^{2+}]_o$ (mM)	pH	Osmolality (mOsm)
Superficial (SZ)	240-270	7-9	6-9	7.1-7.3	310-370
Deep (DZ)	300-350	9-12	14-20	6.6-6.9	370-480
Synovial fluid	140	5	1.5	7.4	280-300

Table 1.1. The ionic environment of articular cartilage.

Table showing the relative extracellular ion concentrations of articular cartilage compared to synovial fluid. The ionic profile of articular cartilage is dependent upon the fixed negative charge on the matrix proteoglycan (PG). Data are cited values from (Maroudas, 1980; Urban, 1994; Wilkins et al., 2000a).

1.2.0 The ionic environment and cartilage hydration.

As previously mentioned, when compared to synovial fluid (or indeed most other human tissues) the ionic environment of cartilage is unusual, with high concentrations of cations (principally Na^+; K^+ and Ca^{2+}) and low concentrations of anions (Cl^- and HCO_3^-; Maroudas, 1980; Wilkins *et al.*, 2000). The concentrations of cations follow the distribution of the proteoglycan, with the SZ having the lowest levels (due to less PG) and the MZ with the highest as a result of PG-fillid collagen 'weave'.

There is also a $[H^+]_o$ difference between the superficial and deep zones of cartilage due to the fixed negative charge carried by the PGs as well as the formation of lactic acid by anaerobic gylcolysis (Stockwell, 1979; Holm *et al.*, 1981). It has been suggested that the waste products of metabolism are removed by diffusion into the synovial fluid (as the calcified zone is highly impermeable and only has a few blood vessels(Maroudas, 1980) and as a result the mid and deep zones are more acidic than the superficial. In femoral head sections from patients 40-50 years of age, the pH of the SZ has been estimated to be 7.1-7.3 and in the DZ the pH is as low as 6.9 (Maroudas, 1980; Wilkins *et al.*, 2000b). Overall, in conjunction with the increased cation concentrations, cartilage has an osmolality in the range of 350-460mOsm.H_2O^{-1} although as previously mentioned, this is dependent upon cartilage zone and FCD (Urban, 1994; Wilkins *et al.*, 2000a).

The hydration and consequently the osmolality of articular cartilage is the therefore inherently linked to the amount of matrix proteoglycan, whose swelling is regulated by the collagen network. Therefore, changes in either the collagen or the PG concentration

will have inherent effects on the mechanical properties of the cartilage. As well as the matrix constituents affecting the cartilage ionic environment, the pressures generated during walking also influence the local cation concentrations and consequently the tissue osmolality. During walking the pressures on articular cartilage cycle from 2atms to 3-5atms (although dependent upon body weight) and this results in the deformation of the cartilage and most likely the chondrocytes contained within (Urban, 1994). If the load is removed immediately the cartilage returns to its 'resting' form. Conversely, if the load is maintained, interstitial fluid is lost (approximately 1-5% v/v per day; (Weightman & Kempson, 1979a; Urban, 1994) from the cartilage proportionally to the pressure applied. This subsequently leads to the effective concentration of PG and a further increase in cation concentration thus raising tissue osmolality (Maroudas & Bannon, 1981). The fluid loss continues until a new equilibrium is reached or until the load is removed (Maroudas & Bannon, 1981; Urban, 1994). Once the load is removed, the cartilage then begins to re-hydrate and the extracellular osmolality eventually returns to non-compressed values (Maroudas & Bannon, 1981; Urban, 1994).

1.3.0 Chondrocytes.

Having reviewed the main constituents of the articular cartilage matrix, attention will now be drawn to the cells that are responsible for their synthesis. Chondrocytes are the only resident cell type in articular cartilage. They mediate the synthesis and breakdown of the extracellular matrix (ECM) in response to the physico-chemical environment yet represent only 1-5% of the tissue mass (human femoral head; Stockwell, 1979; Stockwell, 1991). As previously described, cartilage is often subdivided into three main distinct zones and chondrocytes from each zone differ in size and organisation. Fig 1.1

shows the organisation of the chondrocytes within cartilage, illustrating the unusual ionic environment and the formation of the collagen-PG weave. Fig 1.2 is a confocal image of bovine articular cartilage and clearly shows the three principle zones of articular cartilage

Using observations acquired from bovine cartilage, chondrocytes from the superficial zone are aligned parallel to the cartilage surface, ellipsoid in shape and are approximately 2-3μm height in height with a volume of 396 ± 58μm^3 (Stockwell, 1991; Bush & Hall, 2003). In the mid zone, the chondrocytes are slightly larger and more spherical with a diameter of 8-10μm and a volume of 522 ± 98μm^3 (Stockwell, 1991; Bush & Hall, 2003). Where in the superficial zone the chondrocytes reside in the ECM as single cells, in the mid zone the chondrocytes tend to reside in pairs.

In the deep zone, the chondrocytes are larger still with a volume of 590 ± 21μm^3 (Bush & Hall, 2003). As seen in the MZ, the chondrocytes are grouped usually with six cells per columnar stack arranged perpendicular to the articular surface (Bush & Hall, 2000). It is important to note that this organisation is species dependent. For example, in bovine and equine articular cartilage this arrangement is frequently observed (Bush & Hall, 2001a; Hall & Bush, 2003) where conversely, in human articular cartilage this arrangement is not as easy to distinguish (Bush & Hall, 2003). Within the cartilage, the chondrocytes are surrounded by a specialised pericellular matrix referred to as a chondron and this is described in the next section (section 1.3.1).

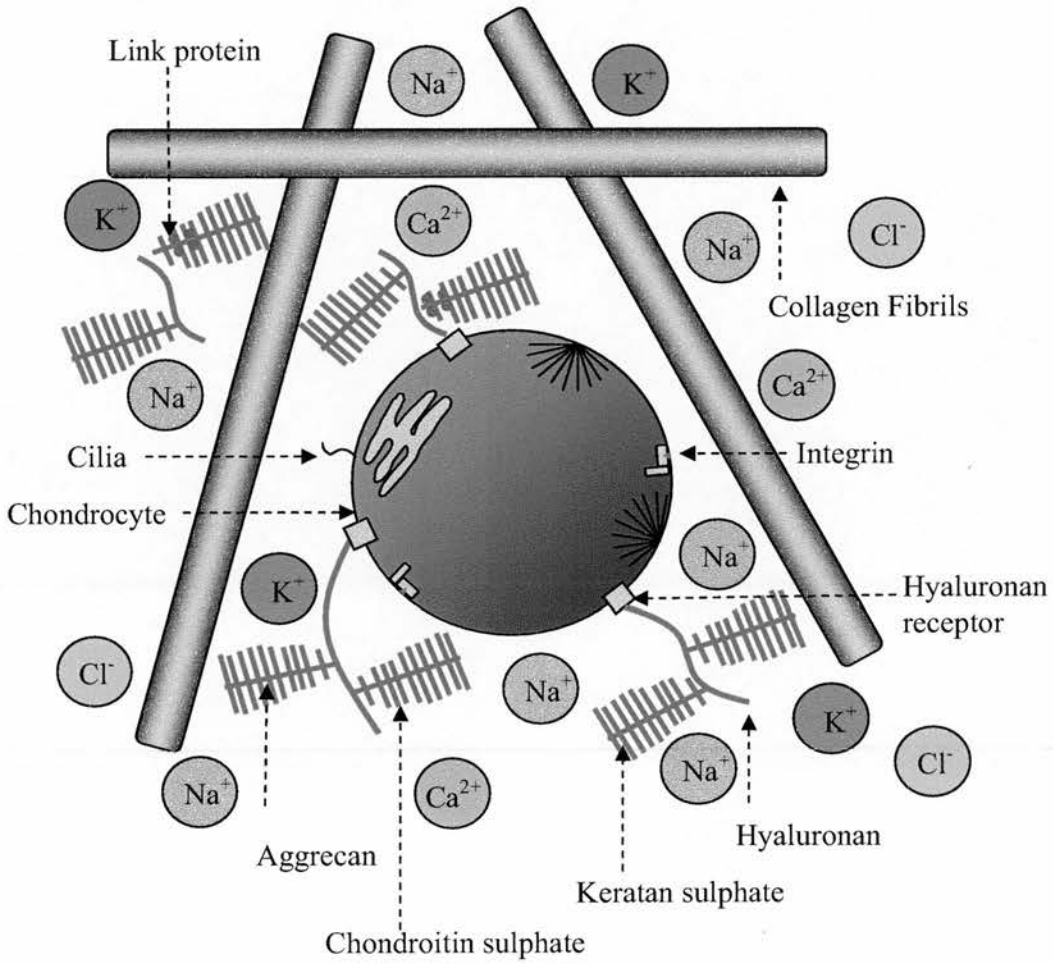

Figure 1.1. The organisation of articular cartilage.

Schematic representation showing the principle physico-chemical environment of articular cartilage. There are higher levels of cations and fewer anions when compared to most other tissues. Not drawn to scale.

1.3.1 Chondrons.

Chondrocytes from all cartilage zones reside in a complex pericellular microenvironment referred to as a chondron (Benninghoff, 1925; Poole *et al.*, 1986, 1987; Poole, 1997). It has been suggested that the chondron forms the primary functional unit of articular cartilage and that the physico-chemical properties of the surrounding microenvironment influence chondrocyte metabolism (Poole, 1997; Hing *et al.*, 2002). Morphologically, a chondron consists of a chondrocyte surrounded by a transparent glycocalyx (rich in proteoglycan and glycoprotein) confined within a fibrillar pericellular capsule (Poole, 1997).

The ECM of the chondron differs in composition to that of the rest of articular cartilage with modifications in the type of proteoglycan and collagen (Poole, 1997). There is a high concentration of sulphated proteoglycan as shown by histochemical studies (Poole *et al.*, 1990) and immunohistochemical studies have shown an increase in aggrecan as well as hyaluronan and link protein (Poole *et al.*, 1991). Of the smaller leucine-rich proteoglycans, decorin was found to be pericellular whereas fibromodulin staining was found to be absent from the chondron (Poole *et al.*, 1993). Consequently, it has been suggested that the chondron has a substantial amount of hyaluronan-aggrecan-link protein complex and that decorin helps to mediate the collagen-proteoglycan interactions (Poole *et al.*, 1991; Poole *et al.*, 1992; Poole, 1997).

As previously mentioned, articular cartilage consists of primarily collagen type II with small amounts of collagen type VI, IX and type XI (Stockwell, 1979; Bruckner &

Vanderrest, 1994; Eyre, 2002). Conversely, collagen type VI is preferentially localised within the pericellular microenvironment of the chondron and interacts directly with the chondrocyte cell surface (Poole *et al.*, 1988; Eyre *et al.*, 1992; Poole *et al.*, 1992). It has been suggested that it is involved in both mediating the structure of the pericellular environment and providing "receptor mediated anchorage and signalling potential between the chondrocyte and the microenvironment" (Poole, 1997). Interestingly, it has been shown during OA that the expression of collagen type VI increases, although there is a loss of the filamentous structure and therefore a potential loss of function (Hambach *et al.*, 1998; Pullig *et al.*, 1999; Swoboda *et al.*, 1999; Soder *et al.*, 2002).

1.3.2 Cilia.

Cilia have been identified in most mammalian tissues and have been found on chondrocytes from mature articular cartilage (Poole *et al.*, 1997). The distal cilia axoneme extends onto the pericellular matrix with a deflection towards the cell surface whereas the proximal end has been shown to associated with the trans face of the Golgi body (Poole *et al.*, 1997). This linkage of the protein synthetic machinery of the cell to the pericellular microenvironment (as the golgi body is involved in protein packaging and targeting) has been suggested as a potential signally mediator in 'sensing' the physiochemical environment (Poole *et al.*, 1985; Poole *et al.*, 1997; Schwartz *et al.*, 1997) and in the deposition of matrix constituents (Poole *et al.*, 1993; Poole *et al.*, 1997). Interestingly, in human osteoarthritic tissue it was found that there was no correlation of cilia structure and distribution between chondrocytes from 'fibrillated and 'non- fibrillated' samples (Kouri *et al.*, 1996).

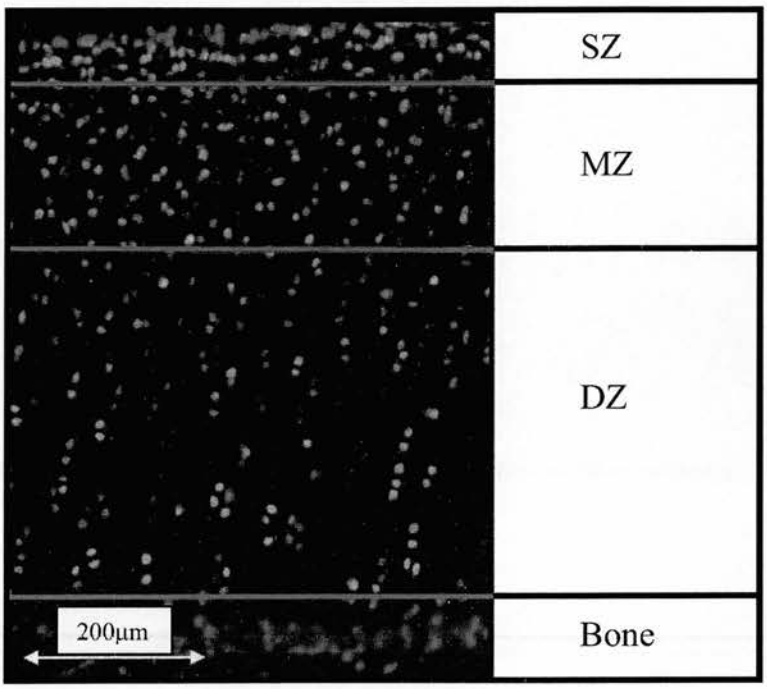

Figure 1.2. Transverse section of bovine articular cartilage.

Transverse section of bovine articular cartilage acquired by confocal microscopy (see Materials and Methods). The organisation of the chondrocytes within the cartilage is clearly visible correlating to the three main cartilage zones. In the superficial zone (SZ) the chondrocytes are ellipsoid and parallel to the articular surface whereas, in the mid zone (MZ), the chondrocytes are more spherical and are often paired. In the deep zone (DZ), the chondrocytes are more spherical and organised into stacks, perpendicular to the articular surface.

1.4.0 Matrix Synthesis.

As the only resident cell type in articular cartilage, chondrocytes are responsible for the synthesis and breakdown of the extracellular matrix (ECM; (Stockwell, 1991). The rate of matrix synthesis is partially regulated by the physico-chemical environment and this permits the adaptation of the ECM to the perceived forces and stimuli as a result of joint use (Kim *et al.*, 1994; Buschmann *et al.*, 1995). Load has a significant effect on both matrix synthesis and composition, while joint immobilisation leads to cartilage thinning and a decrease in PG content (as measured by Safranin O intensity staining; Hung *et al.*, 1997). The mechanism in which chondrocytes are able to 'sense' joint loading is most likely very complex, and one potential signal could be hydrostatic pressure. Work on cartilage explants has shown that hydrostatic pressure within the physiological range (5-15 MPa) influences matrix synthesis (as measured by $[^3H]$ proline and $^{35}SO_4$ incorporation experiments) where pressures above this result in an overall decline in rate (Hall *et al.*, 1990, 1991; Smith *et al.*, 1996; Mizuno *et al.*, 2002).

The signalling pathways involved in the regulation of matrix synthesis are currently unclear, and many factors are involved including changes in extracellular osmolality (Urban *et al.*, 1993; Hopewell & Urban, 2003). As mentioned previously (see section 1.2.0), when static compressive load is applied to cartilage it results in a loss of water from the ECM, an effective concentration of PG and an increase in extracellular osmolality until a new equilibrium is reached or the load is removed (Maroudas & Bannon, 1981; Urban, 1994). Conversely, in response to impact load or in the early stages of osteoarthritis, cartilage overhydrates (and extracellular osmolality decreases)

due to the loss of organisation of the collagen network and the resultant further swelling of the cartilage PG. (Grushko *et al.*, 1989; Guilak *et al.*, 1994; Torzilli *et al.*, 1999). Both an increase or decrease in extracellular osmolality have been shown to influence matrix metabolism, although currently the mechanisms are poorly understood (Urban *et al.*, 1993).

Work on matrix synthesis on *in situ* and freshly-isolated bovine articular chondrocytes have shown that matrix synthesis is at a maximum around ~400mOsm.kg H_2O^{-1}, i.e. the 'generalised' osmolality of the tissue (although does depend upon the zone of cartilage; see section 1.2.0). Deviations from this value resulted in a decrease in synthesis, although if maintained in the aniosmotic osmolality for a period of 8-18 hours chondrocytes were able to adapt and the rate of synthesis increased (Urban *et al.*, 1993). Subsequent work by Hopewell & Urban (Hopewell & Urban, 2003) on alginate encapsulated bovine articular chondrocytes, have shown that after a period of 48 hours chondrocytes maintained in an hypo-osmotic environment continued to have a suppressed level of matrix synthesis, whereas cells in a hyper-osmotic media recovered, and the rate of matrix synthesis actually surpassed control levels. It was shown that the method of adaptation involved the SAPK2 and ERK signalling pathways as the presence of specific inhibitors prevented recovery. Interestingly the ERK pathway has also been implicated in volume 'sensing' as well and regulators of volume sensitive channels, matrix metabolism and modulation of the chondrocyte phenotype (Liedtke & Cole, 2002; Gillis *et al.*, 2001; You *et al.*, 2001; Okazaki *et al.*, 2003n). Surprisingly, in bovine articular chondrocytes the activation of the ERK signalling pathway in response

to fluid flow has been shown to down-regulate aggrecan gene expression (Hung *et al.*, 2000).

As mentioned, during the early stages of osteoarthritis there is an increase in cartilage hydration and consequently a sustained increase in chondrocyte cell volume despite the capacity for Regulatory Volume Decrease (RVD; mechanism for the recovery of cell volume in response to a decrease in osmolality; (Stockwell, 1991; Bush & Hall, 2001b; Bush & Hall, 2003). The increase in hydration is due to the loss of integrity of the collagen weave, and therefore the otherwise restricted PGs are able to swell further. This has a detrimental effect on matrix synthesis that consequently may exacerbate the situation with an increase in pathology. Therefore understanding the mechanisms of cell volume regulation may help in the elucidation of the signalling pathways involved in the regulation of matrix synthesis and the maintenance of ECM integrity.

1.5.0 Chondrocytes and Mechanotransduction.

Due to the complexity of the environment in which chondrocytes reside, the nature of the physical stimuli perceived are difficult to compartmentalise. Compression as the result of joint articulation results in fluid flow, membrane deformation and an increase in extracellular osmolality (Maroudas, 1980; Urban & Hall, 1992; Hall *et al.*, 1996a; Hall, 1999). Conversely, a decrease in extracellular osmolality (for example during OA or as a result impact load[1]; Grushko *et al.*, 1989; Guilak *et al.*, 1994; Torzilli *et al.*, 1999), results in chondrocyte swelling and this in turn may result in membrane stretch and localised fluid flow around the cell. Therefore, a study of a single stimulus may be misleading as all the stimuli are most likely closely linked.

One of the potential mechanisms in which chondrocytes 'sense' changes in their extracellular environment is via membrane stretch and trivalent lanthanides are often used to identify these pathways. Gadolinium (Gd^{3+}) has long be used as an identifier of stretch activated channels (SACs) and in chondrocytes it has been shown to be a suitable inhibitor (Wright *et al.*, 1996; Yellowley *et al.*, 1997; Guilak *et al.*, 1999b). Unfortunately, the use of Gd^{3+} is not without problems. Gd^{3+} readily binds to anions that subsequently sequester it from an experimental saline, including: phosphate, carbonate, EGTA, sulphate, carboxylic acids and albumin (Caldwell *et al.*, 1998). Alternatively, some SACs are in fact Gd^{3+} insensitive including those found in rat astrocytes (Yang & Sachs, 1989) and in snail (*Lymnaea*) neurones (Small & Morris, 1995). Gd^{3+} is also not a specific inhibitor of SAC channels as it has been shown to inhibit both L-type and N-

[1] Impact does not directly result in a change in extracellular osmolality. The resultant decrease in osmolality is due damage of the cartilage-collagen network and the subsequent further swelling of PG.

type calcium channels as well as purine P2X channels (Biagi & Enyeart, 1990; Bleakman *et al.*, 1995). Furthermore, in chondrocyte monolayer culture, Gd^{3+} has been shown to induce apoptosis (Greisberg *et al.*, 2001).

In chondrocytes, mechanotransduction is widely studied, as ultimately matrix synthesis is adapted to signals perceived from the physical (and chemical) environment. Of the potential mechanical stimuli, fluid flow, stretch, membrane deformation and changes in osmolality have been the focus of intense research. Each of these will now be considered in more detail.

1.5.1 Fluid Flow.

Fluid flow is used as a model in chondrocyte mechanotransduction as it mimics shear-induced stress, electrokinetic effects and direct mechanical strain that chondrocytes would experience *in vivo* (Mow *et al.*, 1994; Yellowley *et al.*, 1997). In 2D cultured bovine articular chondrocytes, fluid flow initiates an intracellular calcium ($[Ca^{2+}]_i$) transient mediated by an influx of calcium via a gadolinium-sensitive pathway (Yellowley *et al.*, 1997). The percentage of chondrocytes responding has been shown to increase proportionally to the rate of flow, although the maximal rise in $[Ca^{2+}]_i$ was shown not to change. It was therefore suggested that the amplitude of the $[Ca^{2+}]_i$ was flow-rate independent and consequently an 'all or nothing' response' (Yellowley *et al.*, 1997).

Later work has shown that as well as an influx of calcium, fluid flow also initiates a release of calcium from an IP_3 sensitive store potentially mediated by G-protein

signalling (Yellowley *et al.*, 1999). Interestingly, both pulsate and oscillating fluid flow (pulsate is fluid flow in one direction oscillating flow is in) did not increase the number of responding cells when compared to steady flow, and in fact with an increase in oscillations the number of responding cells actually decreased (Yellowley *et al.*, 1997; Yellowley *et al.*, 1999; Edlich *et al.*, 2001). It was suggested by Edlich *et al.,* that this might be have been due to the viscoelastic properties of the chondrocyte membrane. It was commented by the author (Yellowley *et al.*, 1997; Yellowley *et al.*, 1999; Edlich *et al.*, 2001) that if the fluid oscillations are faster than the viscoelastic time constant of the cell membrane, the increase in flow rate above this would not be 'detected', assuming that membrane deformation is involved in the fluid flow response (Yellowley *et al.*, 1997; Yellowley *et al.*, 1999; Edlich *et al.*, 2001).

1.5.2 Membrane Deformation.

As described above, fluid flow may result in deformation of the chondrocyte membrane. It has been shown that direct mechanical stimulation with a micro-pipette initiates a rise in $[Ca^{2+}]_i$ mediated by an influx of calcium, inhibited by both gadolinium and amiloride (blockers of stretch sensitive anion channels) but not cytochalasin D. Unlike the response to fluid flow, there was no release of calcium from intracellular stores (Guilak *et al.*, 1999b). Interestingly, gadolinium and amiloride where shown to exhibit different affects on the response, with gadolinium decreasing the maximal rise in $[Ca^{2+}]_i$ and amiloride significantly decreasing the number of cells responding. It was suggested that the mechanisms used to detect and subsequently respond to mechanical stress were separate, and that the $[Ca^{2+}]_i$ wave was mediated by a Gd^{3+} sensitive calcium channel and a Ca^{2+}-sensitve K^+ channel and therefore resulting in a self-reinforcing calcium

influx due to the hyperpolarisation of the chondrocyte membrane (Guilak *et al.*, 1999b). If this were found to be true then it would may suggest that the mechanisms for sensing membrane stretch are similar to that of pressure-induced strain; see below (Wright & Salter, 1996; Guilak *et al.*, 1999b).

1.5.3 Pressure-Induced Strain.

Pressure-induced strain experiments have lead to the elucidation of an integrin-mediated, IL-4 mechanotransduction pathway in human articular chondrocytes. In response to intermittent cyclical pressure (0.33Hz; 16Pka; 20mins), the IL-4, autocrine/paracrine, α5β1 integrin receptor-ligand complex is 'activated' and this in turn results in cell hyperpolarisation mediated by a stretch-induced, gadolinium-sensitive low-conductance, apamin-sensitive, Ca^{2+}- activated K^{+} channel (Wright *et al.*, 1996; Wright & Salter, 1996; Wright *et al.*, 1997; Millward-Sadler *et al.*, 1998a; Millward-Sadler *et al.*, 1999). The reduction of $[Ca^{2+}]_o$, the inhibition of L-type calcium channels, and inhibitors of the IP_3 calcium release pathway all decreased the number of responding cells. Interestingly, as seen in response to direct mechanical deformation, gadolinium and amiloride had different affects on the response (Wright *et al.*, 1996; Wright & Salter, 1996; Wright *et al.*, 1997; Millward-Sadler *et al.*, 1998a; Guilak *et al.*, 1999b; Millward-Sadler *et al.*, 1999). Gadolinium inhibited the hyperpolarisation response without a change in basal membrane potential where conversely, amiloride hyperpolarised the chondrocytes and did not block the pressure-strain response until its concentration exceeded 1mmol/L. It was suggested that the hyperpolarisation caused by the amiloride (plus the differences observed in response to direct mechanical

deformation) may be attributed to the inhibition of the Na^{+}/Ca^{2+} exchanger (Wright *et al.*, 1996; Guilak *et al.*, 1999b).

The interleukin-IL-4 pathway has been implicated in the regulation of matrix synthesis in response to pressure-induced strain. Stimulation at 0.33 Hz for 20 mins results in an up-regulation of aggrecan mRNA and the depression of MMP-3 mRNA levels within one hour of stimulation (Millward-Sadler *et al.*, 2000a). The additions of gadolinium and IL-4 antibodies inhibited the response, whereas interestingly, inhibition of the apamin-sensitive K^{+} channels had no affect (Millward-Sadler *et al.*, 2000a). Furthermore, chondrocytes isolated from OA cartilage failed to respond in the same way as chondrocytes isolated from non-degenerate, macroscopically 'normal' cartilage (Millward-Sadler *et al.*, 1998a; Millward-Sadler *et al.*, 2000b). It has been suggested that the IL-4, autocrine/paracrine, a5ß1 integrin receptor-ligand complex is modified by inflammatory mediators (including IL-1 & NO) shown to be up-regulated in OA cartilage (LeGrand *et al.*, 2001) although the cellular basis for this effect is still to be clarified (Millward-Sadler *et al.*, 2000b).

1.5.4 Extracellular Osmolality.

As mentioned previously (see section 1.4.0), changes in extracellular osmolality result in a decrease in matrix synthesis and a transient rise in $[Ca^{2+}]_i$ (Urban *et al.*, 1993; Kerrigan & Hall 2000; Erickson *et al.*, 2001; Kerrigan & Hall 2001; Hopewell & Urban, 2003). The signalling pathways are yet to be elucidated although it has been shown that the regulation of matrix synthesis in response to changes in extracellular osmolality may involve ERK 1 & ERK 2 signalling pathways (Hopewell & Urban, 2003). The rise in

$[Ca^{2+}]_i$ results from an influx from the extracellular environment and may involve the Transient Potential Receptor Cation Channel, V4 (TRPV4; Alford *et al.*, 2003). Furthermore, inhibitors of intracellular calcium store release, G-proteins, the F-actin cytoskeleton and the removal of $[Ca^{2+}]_o$, have all been shown attenuate $[Ca^{2+}]_i$ response (Kerrigan & Hall 2000; Yellowley *et al.*, 2002; Erickson *et al.*, 2001; Erickson *et al.*, 2003; Pritchard *et al.*, 2002) although currently there is no direct link to the changes in $[Ca^{2+}]_i$ and the capacity for volume regulation.

In response to both hyper- and hypo-osmolality, chondrocytes are able to volume regulate by Regulatory Volume Increase (RVI) and Regulatory Volume Decrease (RVD) respectively (although it should be noted the capacity for RVI has been shown to be in response to the 'post RVD-RVI' protocol; (Errington & Hall, 1995; Bush & Hall, 2000; Kerrigan & Hall, 2000a; Yellowley *et al.*, 2002). It is still not clear if the rise in $[Ca^{2+}]_i$ is required to mediate volume regulation, although it has been shown that the attenuation of the $[Ca^{2+}]_i$ rise decreased the number of chondrocytes able to volume regulate by RVD (Kerrigan & Hall, 2000). Interestingly, it has been shown that further RVI in bovine articular chondrocytes can be stimulated by the removal of $[Ca^{2+}]_o$ and consequently the inhibition of the $[Ca^{2+}]_i$ rise. This mechanism is not clear but it has been suggested by the author that the removal of $[Ca^{2+}]_o$ influences chondrocyte membrane integrity or Ca^{2+} sensitive microvilli (Erickson *et al.*, 2001).

Having reviewed some of the mechanisms of mechanotransduction in chondrocytes, it is apparent that there are similarities and differences between each of these responses. These are summarised in Table 1.2. One of the most noticeable differences is the

dependence upon the actin cytoskeleton. Pressure-induced strain is dependent upon a polymerised actin cytoskeleton and the induced hyperpolarisation is inhibited by the addition of cytochalasin D (2μM; assuming the inhibition is not through the cytochalasin D directly inhibiting the channel activity; Wright *et al.*, 1996; Wright & Salter, 1996; Wright *et al.*, 1997; Millward-Sadler *et al.*, 1998a; Millward-Sadler *et al.*, 1999; Millward-Sadler *et al.*, 2000b; Salter *et al.*, 2001). Conversely, the responses to direct mechanical deformation and changes in extracellular are not inhibited by the depolymerisation of the actin cytoskeleton (Guilak *et al.*, 1999a; Guilak *et al.*, 1999b). This would seem to imply that the IL-4, autocrine/paracrine, α5β1 integrin receptor-ligand complex may not be a mediator involved in either volume regulation or indeed direct membrane stretch although the involvement of the actin cytoskeleton is not yet completely understood. Another potential overlap of the described mechanosensory mechanisms is between membrane stretch and pressure-induced strain. As mentioned previously, the calcium rise (or subsequent oscillations) in response to membrane deformation may be potentiated by a the activity of the Ca^{2+}-sensitive K^+ channel activated in response to pressure-induced strain. As there are differences on the dependence of an intact F-actin cytoskeleton, it is possible that the pathways share a common theme although still inpart be independent.

Of interest is the fact that all the pathways described seem to involve stretch sensitive channels as shown by sensitivity to Gd^{3+}, although as previously mentioned, Gd^{3+} has been shown to affect other cellular processes (section 1.5.0). Despite the noted differences, there does appear to be an overlap in the methods of 'sensing' changes in

the physico-chemical environment and these differences are most likely required to 'fine-tune' the chondrocyte response.

Observation	Fluid Flow	Mechanical stimulation	Pressure-induced strain	Osmotic change
Calcium influx	YES - EGTA prevents the $[Ca^{2+}]_i$ rise.	YES – EGTA prevents the $[Ca^{2+}]_i$ rise.	YES - inhibited by L-type Ca^{2+} channel blockers plus EGTA.	YES – Via the TRVP4 cation channel? EGTA prevents the $[Ca^{2+}]_i$ rise.
Membrane stretch receptors	YES – inhibited by gadolinium (both no. cells responding and peak $[Ca^{2+}]_i$ rise).	YES – inhibited by both Gadolinium (Peak $[Ca^{2+}]_i$ change) and Amiloride (no. cells responding).	YES – inhibited by gadolinium. Amiloride caused dose-dependent hyper-polarisation. Inhibited at 1mmol/L.	YES – inhibited by gadolinium (2D cultured cells not freshly isolated).
Intracellular calcium stores	YES - Blocked by neomycin and thapsigargin not by ryanodine or caffeine.	NO - Not inhibited by dantrolene, thapsigargin	YES - Not inhibited by thapsigargin. Inhibited by PLC antagonists.	YES – attenuated by thapsigargin (2D cells). Not inhibited by dantrolene (freshly isolated).
G-protein interactions	Inhibited by pertussis toxin.	******	******	******
Involement of ATP	NO	******	NO	******
Cytoskeletal involvement	******	NO – not inhibited by Cytochalasin D.	YES –inhibited by Cytochalasin D.	YES- Hypo, results in F-actin breakdown. Hyper, no change.
Membrane deformation	The use of quartz coverslips did not inhibit the response	YES – response inhibited using quartz glass.	YES – response inhibited using glass.	NO - membrane deformation not enough to stimulate $[Ca^{2+}]_i$ rise.

Table 1.2. Mechanosensing in chondrocytes.

*The table on the previous page summarises the four types of mechanical stimulation previous discussed (see section 1.6.0). It is interesting to note that there is a large degree of overlap between the mechanical stimuli although there are notable and rather significant differences. There appeared to be a difference on the dependence upon the intact F-actin cytoskeleton where both RVD and direct mechanical deformation appear to be independent where conversely it is required to mediate the pressure-induced strain response. All stimuli appear to initiate a rise in $[Ca^{2+}]_i$ and in all cases excluding direct stimulation there is a contribution from cellular stores. The last major difference is in the response to membrane deformation. Responses to direct mechanical stimulation and pressure-induced strain were inhibited by performing the experiments quartz coverslips where conversely this had no effect on the response to fluid flow. It was suggested that ATP could be involved in mechanosensing of fluid flow as an autocrine/paracrine agent or direct membrane perturbation through ATP-sensitive K^+ channels. The addition of drugs know to inhibit these pathways (cromakalim or suramin) has no effect and therefore it was concluded that ATP was not involved. To summarise, despite the overlap in the chondrocyte response to certain mechanical stimuli there are differences in the responses and it is these differences that may allow for the 'fine-tuning' of the response. ****** Denotes not tested to date.*

Reference:

Fluid flow:
Yellowley *et al.*, 1997; Yellowley *et al.*, 1999; Edlich *et al.*, 2001.

Mechanical stimulation:
Guilak *et al.*, 1999a; Guilak *et al.*, 1999b.

Pressure-induced strain:
Wright *et al.*, 1996; Wright & Salter, 1996; Wright *et al.*, 1997; Millward-Sadler *et al.*, 1998a; Millward-Sadler *et al.*, 1999; Millward-Sadler *et al.*, 2000b; Salter *et al.*, 2001.

Osmolality:
Errington & Hall, 1995; Bush & Hall, 2000; Erickson *et al.*, 2001; Kerrigan & Hall, 2000; Yellowley *et al.*, 2002.

1.6.0 Regulatory Volume Decrease (RVD)

As previously mentioned, ^{35}S-Sulphate incorporation experiments on *in situ* and isolated chondrocytes have shown that matrix synthesis is sensitive to changes in extracellular osmolality (Urban *et al.*, 1993; Hopewell & Urban, 2003) and possibility a change in cell volume. The need for effective volume regulation has been identified in chondrocytes as well as a wide range of other cell types, as a prerequisite for optimal cellular metabolism (Lang *et al.,* 1998), and it has been suggested that intracellular K^+ concentration may also be important (Urban 1994). In response to a decrease in osmolality, a variety of animal cells are able to regulate their volume back towards their initial volume set-point, by a process termed Regulatory Volume Decrease; (RVD; Hoffmann & Pedersen, 1998; Hoffmann & Mills, 1999; O'Neill, 1999; Hoffmann, 2000).

RVD results in the loss of intracellular osmolytes (with associated water) and consequently the recovery of cell volume towards the initial set-point. There are several transporters that are potentially capable of mediating this response including: (1) Large conductance Ca^{2+} activated K^+ channels; (2) K^+ channels; (3) K^+ Cl^- cotransporter (KCC); (4) Cl^- channels and (5) a Volume Sensitive Anion Channel (VSAC; O'Neill 1999; Hoffmann, 2000; Fig. 1.3). Depending upon the cell type, the chorus of transporter(s) used for RVD are different and therefore each transporter will be briefly described in turn. Finally, the current literature on RVD in chondrocytes will be reviewed.

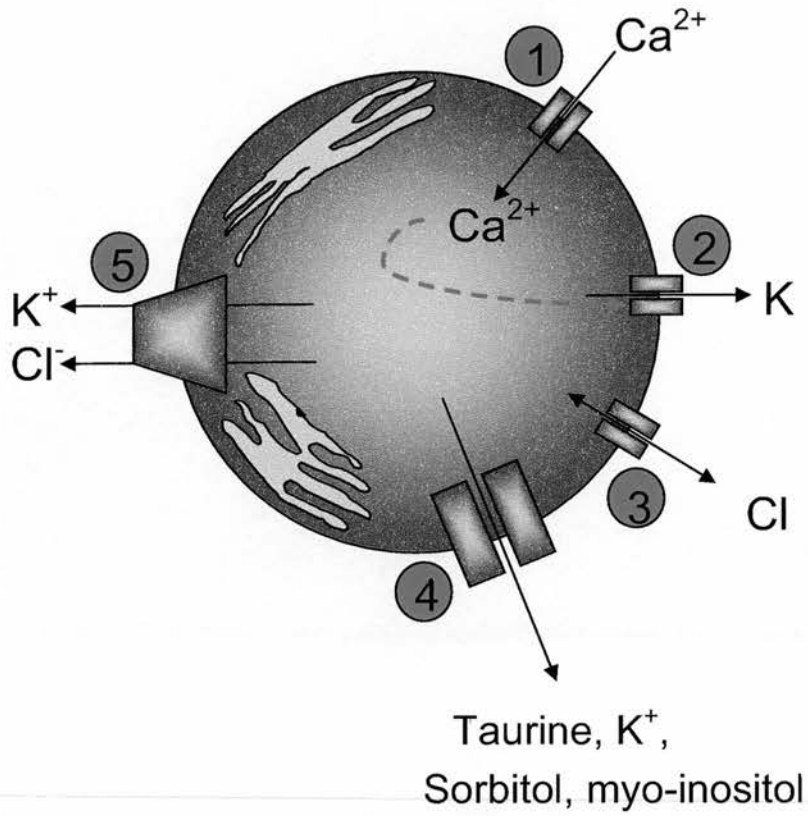

Figure 1.3. Diagrammatic representation of the membrane transporters involved in cell RVD.

The above diagram shows the principle membrane transporters known to be used to mediate RVD in an 'a typical' cell. (1+2) Ca^{2+}- activated K^+ channels (3) Cl^- channels (4) Volume Sensitive Anion channel (VSAC) and (5) K^+- Cl^- cotransporter (KCC).

1.6.1 Potassium Channels

One of the main types of channel involved in RVD are the large conductance Ca^{2+} activated K^+ channels (also known as BK channels; O'Neill 1999). BK channels (a member of the calcium activated - K^+ channel super family) were first cloned from the fruit fly *Drosophila Melanogaster* in 1991 (Atkinson *et al.*, 1991; Weiger *et al.*, 2002) are characterised by a large conductance of 200-300 pS (Weiger *et al.*, 2002) and have been the subject of intense study due to their potential involvement in disease (Vergara *et al.*, 1998). They are almost ubiquitously expressed (although not present in cardiac myocytes (Toro *et al.*, 1998) and highly conserved within, and between species (Toro *et al.*, 1998; Shipston *et al.*, 1999; Shipston, 2001; Weiger *et al.*, 2002). They are encoded by a single gene (Slo) with splice variants that provide functional diversity (Vergara *et al.*, 1998).

The role for K^+ efflux in volume regulation was first experimentally shown by Roti-Roti & Rothstein (Roit-Roti & Rothstein, 1973) and further supported by Hendil & Hoffman (Heindil & Hoffmann, 1974). Using lymphocytes and Ehrlich tumour cells respectively, it was found that in response to a hypo-osmotic challenge, there was an activation of a K^+ leak efflux and therefore an assumed recovery in cell volume. Later work has shown that K^+ maxi channels are also involved in RVD in human bronchial epithelia (HBE) cells (Fernandez-Fernandez *et al.*, 2002) and immortalised human osteogenic precursor cells (Weskamp *et al.*, 2000b). In both cell types, the channels are activated by a rise in intracellular calcium and changes in voltage. Interestingly, the RVD response in the HBE cells is inhibited by gadolinium (inhibitor of stretch sensitive channels) and thought to involve the Transient Potential Receptor Cation Channel (TRPV4; shown by

immunohistochemistry). Recently, this channel has also be shown to be expressed in chondrocytes (Alford *et al.*, 2003) and is thought to mediate the rise in $[Ca^{2+}]_i$ in response to changes in osmolality.

As well as the large conductance K^+ channels, small conductance voltage activated K^+ channels have also been implicated in RVD (Hoffmann & Mills, 1999) including the Kv 1.3 (n-type) and Kv 1.5 K^+ channels. In human lymphocytes, both n-type charybdotoxin sensitive K^+ channels and charybdotoxin insensitive K^+ channels mediate RVD where conversely in rat hepatocytes only the latter are involved (Grinstein & Smith, 1990; Jenkinson & Sandford, 1990; Sandford *et al.*, 1992). Unlike the BK channels, the small conductance K^+ channels are not activated by an increase in $[Ca^{2+}]_i$ *per se*, instead membrane depolarisation as a result of the calcium sensitive chloride channels (CaCCs; see later) initiates the K^+ efflux.

1.6.2 Potassium Chloride cotransporter (KCC)

The KCC cotransporter was first identified by swelling red blood cells in hypo-osmotic solutions and then treatment with N-ethylamleimide (NEM; a stimulator of the channel; (Lauf & Theg, 1980; Lauf & Adragna, 2000). The KCC is a member of the cation-chloride cotransporter family and characterised as a low conductance, low affinity, electroneutral cotransporter ubiquitously expressed in most cell types (Lauf & Adragna, 2000). To date four KCC genes have been identified giving rise to 4 different isoforms (termed KKC1 to KKC4) each with different functions and in some cases distribution (Roussa *et al.*, 2002; Shen *et al.*, 2001; Isenring & Forbush, 2001; Lauf & Adragna, 2000; Lauf *et al.*, 2001).

The KCC1, KCC3 and KCC4 isoforms are sensitive to changes in osmolality and potentially involved in the RVD response (Lauf *et al.*, 2001; Shen *et al.*, 2001). The KCC1 isoform has been studied in more detail and it has been shown that of the 12 transmembrane domains, a deletion of the c-terminus resulted in a loss of activation and hence it has been suggested to be involved in signal transduction (Lauf *et al.*, 2001). In sheep erythrocytes, it has been shown that the activity of the KCC1 cotransporter is regulated by the volume sensitive, calcium dependent myosin light chain kinase (MLCK) and inhibited by the kinase inhibitor (ML-7; although stimulatory in response to hypotonicity; Kelley *et al.*, 2000). Further work on rat vascular smooth muscle cells has shown that the amount of mRNA and consequently cotransporter expression for both isoforms is regulated by cGMP- dependent protein kinase G (Di Fulvio *et al.*, 2001a; Di Fulvio *et al.*, 2001b). Conversely, the exact role for the KCC3 isoform is currently less clear although it has thought to be involved in cell proliferation (Shen *et al.*, 2001).

Unlike the other three isoforms, the KCC2 is not activated by cell swelling although is stimulated by NEM (Payne, 1997). It is only expressed in the brain and in particular neurons of the cortex, hippocampus and the cerebellar granular layer (Lauf & Adragna, 2000). It has been shown to mediate a reduction in the intracellular Cl^- concentration and thus resulting in cellular hyperpolarisation via GABA and glycine-mediated Cl^- channels (Jarolimek *et al.*, 1999). Interestingly it shares a 69% sequence homology with KCC4 and a 71% homology with KCC2 despite these differences in function and distribution (Lauf & Adragna, 2000).

1.6.3 Chloride Channels.

Chloride channels are classified into five main families based on channel gating. These include: (1) voltage-dependent/gated Cl^- channels (CIC), (2) calcium activated calcium channels (CaCC), (3) volume sensitive anion channel (VRAC), (4) protein kinase/nucleotide mediated (CFTR) and (5) ligand activated (GABA). In mammalian tissues, the CIC family consists of nine members coded for by nine genes (Nilius & Droogmans, 2003). Of the nine CIC members, it has been shown that CIC2 and CIC3 (both ubiquitously expressed are activated by changes in cell volume Nilius & Droogmans, 2003) although interestingly the expression of CIC3 on the plasma membrane is requires further classification. Cell swelling initiates an outward rectifying, medium conductance Cl^- current in numerous cell types and coupled to the loss of K^+ (therefore helping to maintain membrane potential) subsequently brings about RVD (Okada, 1997; Hoffmann & Simonsen, 1989; O'Neill, 1999; Hoffmann, 2000; Nilius & Droogmans, 2003).

The calcium activated chloride channels (CaCCs) are expressed in both excitable and non-excitable cells have been identified in numerous cells types including epithelial, endothelial, neuronal and cardiac myocytes (Nilius *et al.*, 1997b; Nilius *et al.*, 1997c; Nilius *et al.*, 1997e). The channels are characterised by a small conductance of 0.5-5pS (compared to ~40pS of the CIC3) and activity is associated with a voltage-dependent increase in $[Ca^{2+}]_i$; Nilius & Droogmans, 2003). A change in extracellular osmolality initiates a rise in $[Ca^{2+}]_i$ in many cell-types from an influx from the extracellular environment and in some cell types a release from intracellular stores (Hoffmann & Mills, 1999; Jakab *et al.*, 2002). This rise in $[Ca^{2+}]_i$ in turn activates CaCC (mediated by

Ca^{2+} binding to the channel; Nilius & Droogmans, 2003) that leads to an efflux of Cl^- and in conjunction with K^+ channels subsequent RVD.

Of the last families within the chloride channel super-family, CFTR and GABA are not involved in volume regulation whereas conversely, the VSAC is. The involvement of this last channel type is discussed below.

1.6.4 Volume Sensitive Anion Channel (VSAC).

The VSAC has a single-channel conductance of 50-90pS (when held at a depolarising voltage; (Nilius & Droogmans, 2003) and this can be carried by various anions (in sequence of permeability): $SCN^- > I^- > NO_3^- > Br^- > Cl^- > F^-$ (termed Eisenmann's sequence 1; (Nilius *et al.*, 1994b; Nilius *et al.*, 1997f; Nilius & Droogmans, 2003). In endothelial cells, it has been approximated using the 'excluded volume model' that the channel has a diameter of ~ 11Å (0.73 ± 1.1nm and 1.15 ± 4.8nm; Nilius *et al.*, 2000; Nilius & Droogmans, 2003) and therefore permeable to amino acids (including taurine, glycine and aspartate), polyols (including sorbitol and myo-Inositol), methylamines (including betaine and glycerol-phosphorylcholine), lactate and bicarbonate (Junankar & Kirk, 2000; Nilius *et al.*, 2000; Nilius & Droogmans, 2003). The VSAC is also permeable to ATP and therefore as well as being regulated by ATP itself (through the binding of the nucleotide to the channel or a regulatory protein and not by hydrolysis; Nilius & Droogmans, 2003) it is also involved in the secretion of ATP involved in autocrine/paracrine signalling (Oike *et al.*, 1994; Sabirov *et al.*, 2001).

To date, the molecular identity of the VSAC has not been elucidated and this is partly attributed to the lack of a specific inhibitor (Nilius *et al.*, 2000; Nilius & Droogmans, 2003). Initial work has shown that the volume sensitive Cl^- efflux is sensitive to the classic Cl^- channel blockers including DIDS, NPPB, SITS, as well as some more unexpected inhibitors including Tamoxifen (Nilius *et al.*, 1994a), glibenclamide (IC_{50} 261.0 ± 8.4μM; Liu *et al.*, 1998b), and mefloquine (IC_{50} 1.19 ± 0.07μM; Maertens *et al.*, 2000). Candidates for the molecular identity of the VSAC have been proposed including CIC3, pI_{Cln} and P-glycoprotein (product of the multi resistance gene 1; Li *et al.*, 1998). CIC3 has been ruled out as it has a different pharmacological and biophysical profile (Nilius *et al.*, 1997a; Nilius *et al.*, 1997b; Nilius *et al.*, 1997d) although its role is currently being re-reviewed (Hermoso *et al.*, 2002). pI_{Cln}, a 27kDa protein cloned from the *Madin Darby Canine Kidney* cell library is an unlikely candidate as it is more selective to cations than anions (as shown in bilayer experiments; Li *et al.*, 1998) is principally cytosolic and does not localise to the plasma membrane upon hypo-osmotic stimuli (Emma *et al.*, 1998; Furst *et al.*, 2000; Nilius & Droogmans, 2003). Finally, P-glycoprotein seems an unlikely candidate as it has been shown to have a different pharmacological profile, there with no correlation between current and channel density (Nilius & Droogmans, 2003). Furthermore, it has been reported that anti-sense oligonucleotides failed to inhibit VSAC activity (Tominaga *et al.*, 1995).

1.6.5 Potential RVD Transporters in Chondrocytes.

Having reviewed the principle channels involved in the RVD response, examining the expression of these channels/transporters in chondrocytes would help in the understanding of the chondrocyte RVD response. As mentioned previously one of the

major pathways of the RVD involves the KCC whose activity is dependent upon the presence of Cl^- (Lauf & Adragna, 2000). In freshly-isolated chondrocytes it has been shown in response to a decrease in extracellular osmolality the volume activated ouabain- and bumetanide-insensitive K^+ efflux was not inhibited by the substitution of Cl^- with NO_3^- (Hall *et al.*, 1996b). This would imply that the KCC is not a mediator of the chondrocyte RVD response (whose activity is dependent upon Cl^-; Hall *et al.*, 1989) although further work has shown that NEM induced a slight stimulation although latent under normal experimental conditions (Hall *et al.*, 1996b).

The presence of voltage activated potassium channels have been shown in chondrocytes from numerous species including: rabbit, porcine, avian and human (Grandolfo *et al.*, 1992; Walsh *et al.*, 1992; Mozrzymas *et al.*, 1994; Sugimoto *et al.*, 1996; Wright *et al.*, 1996; Martina *et al.*, 1997; Mozrzymas *et al.*, 1997). Both the large conductance K^+ channel (BK) identified in porcine articular chondrocytes and the small conductance calcium activated K^+ channels (SK) found in human chondrocytes are activated by membrane stretch and inhibited by tetraethylammonium (TEA; a K^+ channel blocker; Wright *et al.*, 1996; Martina *et al.*, 1997; Mozrzymas *et al.*, 1997). Conversely, the K^+ channel identified in avian growthplate chondrocytes is insensitive to TEA although is inhibited by charybdotoxin and 4-aminopyridine (two different types of K^+ channel blocker; Walsh *et al.*, 1992). Later work on the K^+ transport in avian growthplate chondrocytes revealed the presence of a large conductance calcium activated K^+ channel, sensitive to TEA and protein kinase A (PKA). This subsequent pharmacological data is consistent with Maxi K^+ channel activity and may indicate the

presence of two types of large conductance K^+ channel in avian growthplate chondrocytes (Long & Walsh 1994).

The presence of Cl^- channels have been shown by a series of patch clamp experiments measuring changes in chondrocyte membrane potential. Experiments on rabbit articular chondrocytes have shown the presence of an outwardly rectifying current, whose reversal potential is close to that of Cl^- (Sugimoto *et al.*, 1996). The channel is sensitive to 4-acetamido-4'- isothiocyanostilbenezene-2, 2-disulfonic acid (SITS; a Cl^- channel blocker) although not inhibited by DIDS (Sugimoto *et al.*, 1996). Subsequent work on porcine articular chondrocytes has shown that the resting membrane potential is mainly dependent upon the activity of Cl^- channels as changes in both intracellular and extracellular Cl^- concentration alter the potential following Nernst equation predictions (Tsuga *et al.*, 2002). Interestingly, in bovine articular chondrocytes it has been suggested that the change in membrane potential as a result of a hypo-osmotic challenge is mediated by a Cl^- efflux with similar kinetics to that descried by Sugimoto *et al* (Sugimoto *et al.*, 1996; Yellowley *et al.*, 2000; Yellowley *et al.*, 2001; Yellowley *et al.*, 2002).

As the molecular identity of the VSAC has not been elucidated, its expression in chondrocytes has not been conclusively proven. Work on bovine articular chondrocytes has shown that in response to a decrease in extracellular osmolality an efflux of organic osmolytes (including taurine, sorbitol and myo-inositol) as well as K^+ and Cl^- is initiated (Hall, 1994; Hall *et al.*, 1996b; Hall & Bush, 2001; Yellowley *et al.*, 2002). The efflux (and consequently RVD) can be inhibited using pharmacological agents known to block

the VSAC, although not all inhibitors work, and some of the known stimulators (including arachidonic acid, Lipoxin A_3 & B_3 and leukotrines $LTB/C/D_4$) also had little effect (Hall, 1995; Hall & Kerr, 2000; Kerrigan & Hall, 2000; Hall & Bush, 2001). This therefore suggests that the anion channel used to mediate RVD in chondrocytes may not be the VSAC observed in other cell types.

1.6.6 RVD in articular chondrocytes.

Having reviewed the known functional RVD channels/transporters present in chondrocytes, the literature on the capacity for RVD will be summarised. In articular chondrocytes, although the signalling mechanisms are poorly understood, it has been shown in response to an acute hypo-osmotic challenge that chondrocytes are able to volume regulate by RVD (Hall *et al.*, 1995; Bush & Hall, 2000; Kerrigan & Hall, 2000). The nature of the channel(s) involved are yet to be defined although it is thought that RVD is principally mediated through a highly permeable, low-selectivity 'osmolyte channel' (Hall *et al.*, 1996a; Hall & Bush, 2001). As of yet the mechanism(s) in which chondrocytes perceive a change in cell volume is/are unknown although potential stimuli may include:

- Changes in intracellular ionic composition
- Unfolding of the plasma membrane
- Macromolecular crowding
- Membrane stretch/deformation
- Pressure-induced strain
- Changes in the actin cytoskeleton

- G-proteins
- Changes in $[Ca^{2+}]_i$.
- Intracellular pH

In response to an osmotic challenge (280mOsm.kg H_2O^{-1} to 180mOsm.kg H_2O^{-1}), *in situ* chondrocytes swell by approximately 16% with no discernable difference between the three main cartilage zones (SZ, MZ, DZ; Bush & Hall, 2000; Bush & Hall, 2001a). Chondrocytes are then able to perform RVD such that 20 mins after the osmotic challenge, volume is recovered to within 3% of the initial volume (Bush & Hall, 2001a). The capacity for RVD was compared between *in situ* and freshly-isolated chondrocytes and it was found that despite the isolated cells having a larger 'resting volume' (735 ± 73.2 μm^3 compared to 552 ± 36.3 μm^3; MZ) no significant difference in either the extent of cell swelling or the rate of RVD existed (Bush & Hall, 2000; Bush & Hall, 2001a). In fact, later work has shown that irrespective of the isolation osmolality (180/280/380mOsm.kg H_2O^{-1}) volume stabilises at ~20 hours post-isolation to a similar 'set-point' value (Hall & Bush, 2001). This regulation of set-point has been observed in other cell types including red blood cells, EAT cells and C6 glioma cells (Starke & McManus, 1988; Lauf, 1991; Emma *et al.*, 1997; Hoffmann, 2000; Joiner *et al.*, 2002).

In isolated and *in situ* chondrocytes a decrease in extracellular osmolality results in a volume-sensitive taurine efflux (as measured by ^{14}C[taurine]), where the extent of the response is proportional to the osmotic challenge (Hall, 1995; Hall & Bush, 2001). Interestingly when comparisons were performed between isolated and *in situ* chondrocytes, for a given osmotic challenge no significant difference was observed therefore implying that interactions with the ECM are not mediators of the response and

that the volume 'sensor' probably resides within (or on) the cell (Bush & Hall, 2000; Hall & Bush, 2001; Bush & Hall, 2001a).

The molecular identity of the 'osmolyte channel' channel has not been confirmed due to a complex pharmacological profile. It is thought that the 'osmolyte channel' is a channel as opposed to a carrier-mediated pathway as: (1) the channel has a low specificity as it is also permeable to uridine, sorbitol and inositol; (2) the addition of substrate on the trans-membrane side did not stimulate the pathway, i.e. no transmembrane stimulation (Miller, 1968; Stein, 1986; Hall, 1995; Carrauthers & Zottola, 1996) had no effect on taurine transport and (3) the action of the taurine transport was linear and followed Michaelis – Menton saturation kinetics (Hall, 1995). Later work by Bush & Hall (Bush & Hall, 2000) have shown that chondrocytes are able to volume regulate at room temperature therefore suggesting that there is little (if any) translocation of the channel from an intracellular store to the plasmas membrane as this process is temperature sensitive (Bush and Hall, 2000a).

It has been shown that the volume induced taurine efflux can be inhibited by numerous drugs including some the classic Cl^- channel blockers including: DIDS; NPPB and MK 196 as well as inhibitors shown to block the VSRC including: quinine; tamoxifen and NDGA (Hall, 1995; Hall & Bush, 2001; Nilius & Droogmans, 2003). Of all the inhibitors tested to date, the most the most effective has been shown to be REV 5901 (50μM), a competitive inhibitor of the LTD_4 receptor and 5-lipoxygenase inhibitor (Khandwala *et al.*, 1985; Aharony *et al.*, 1986). Both pre-incubation and application during the hypo-osmotic challenge have been shown to inhibit the taurine efflux with

equal efficacy (~94% inhibition) although interestingly more specific inhibitors of the arachidonic acid cascade and leukotrine LTD_4 release failed to significantly inhibit RVD (Hall, 1995; Hall & Kerr, 2000; Kerrigan & Hall, 2000; Hall & Bush, 2001). It has therefore been postulated that REV 5901 is acting as a broad, non-specific inhibitor of an 'osmolyte channel', and that RVD, unlike in EAT and HEC cells, does not involved steps of the arachidonic acid cascade (Nilius *et al.*, 1994a; Hall & Kerr, 2000; Hoffmann & Hougaard, 2001).

The taurine concentration of *in situ* chondrocytes has been estimated to be 1.8 ± 0.09 mmol (1 cell water)$^{-1}$ and therefore unlikely to be the main component of the RVD response (Hall, 1995). Later work has shown that a decrease in osmolality also initiates an efflux of K^+ via an ouabain- and bumetanide-insensitive, pimozide sensitive pathway (Hall *et al.*, 1996b). The efflux is rapid in activation and sensitive to small changes in osmolality being stimulated by as little as a ~9% decrease in osmolality (Starks & Hall, 1995; Hall *et al.*, 1996a; Hall *et al.*, 1996b). Subsequent work on cultured bovine articular chondrocytes has shown that the decrease in osmolality results in a gadolinium-sensitive, outwardly rectifying current and it has been calculated that the current is mainly attributed to the efflux of Cl^- and only partially as a result of K^+ (Yellowley *et al.*, 2000; Yellowley *et al.*, 2001; Yellowley *et al.*, 2002).

In summary, articular chondrocytes have the capacity for RVD in response to a decrease in osmolality. The osmotic challenge results in a volume sensitive efflux of taurine through an unidentified channel, although there are some pharmacological similarities

to both Cl^- channels and the VSRC. A hypo-osmotic challenge also results in the efflux of K^+ and potentially Cl^- although the exact mechanism is still to be elucidated.

1.7.0 Regulatory Volume Increase.

Cellular homeostasis and in particular cell volume is important in the maintenance of metabolic function and the viability of a cell (Wright & Rees, 1998). Deviations from the set-point volume have been shown in many cell types to affect metabolism and this is also true in chondrocytes (Urban, 1994). Chondrocytes are responsible for the synthesis of the extracellular cartilage matrix and it has been shown that changes in extracellular osmolality decrease matrix synthesis as measured by radioactive sulphate incorporation (Urban *et al.*, 1993). A decrease in extracellular osmolality results in a depression of matrix synthesis where conversely a sustained hyper-osmotic environment initially results in a depression of synthesis that is subsequently recovered and then surpasses the initial 'control' rate (Urban *et al.*, 1993; Hopewell & Urban, 2003).

In order to limit the potential deleterious effects as a result of changes in extracellular osmolality, cells have developed volume regulatory pathways designed to maintain the cell at a pre-determined volume 'set-point' (Hoffmann & Pedersen, 1998; Hoffmann & Mills, 1999; O'Neill, 1999; Hoffmann, 2000). However, in chondrocytes how the 'set point' is determined or indeed regulated (as it has been shown to be amendable; Hall & Bush, 2001) is currently unknown. It is known for a given environmental state, the 'set-point' is the volume that the cell volume regulates towards (where possible) should there be a change in volume (Hall *et al.*, 1995).

In response to an increase in extracellular osmolality, volume is often recovered by a process termed Regulatory Volume Increase (RVI). This pathway adjusts the intracellular milieu of the cell by initiating a net influx of ion/cations and other

osmolytes that consequently result in the influx of osmotic water and the restoration of cell volume. The increase in intracellular osmolality is mediated by a set of membrane transporters, principally: (1) Na^+-K^+-$2Cl^-$ cotransporter (NKCC); (2) Na^+/H^+ exchanger (NHE) coupled to the Cl^-/HCO_3^-; (3) Na^+-K^+ pump; see Figure 1.4. (4) Na^+/HCO_3^- cotransporter (Wilkins *et al.*, 2000b). Depending upon the cell type, and indeed the type of osmotic increase (iso-osmotic or hyper-osmotic), either a single type of transporter or a chorus of them all may be used to mediate the recovery in volume. Due to the complexity of the RVI response each of the membrane transporters will be reviewed in turn.

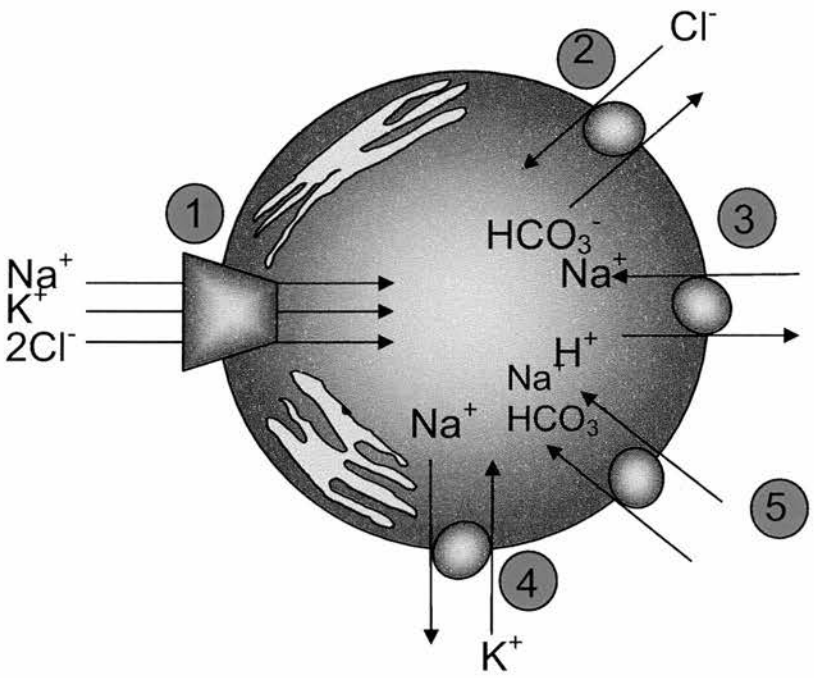

Figure 1.4. Diagrammatic representation of the membrane transporters involved in cell RVI.

The above diagram shows the membrane transporters known to be used to mediate RVI in an 'a typical' cell. (1) Na^+-K^+-2Cl^- cotransporter (2) HCO_3^-/Cl^- exchange (3) Na^+/H^+ exchange (4) Na^+-K^+ pump. (5) Na^+/HCO_3^- coupled cotransporter. Adapted from O'Neill (1999).

1.7.1 Na^+-K^+-$2Cl^-$ cotransporter

The Na^+-K^+-$2Cl^-$ (NKCC) is an electroneutral, bidirectional cotransporter belonging to the Cation–Chloride Cotransporter family (CCC; Lauf *et al.*, 1987; Isenring & Forbush, 2001; Flatman, 2002). It exists in two isoforms referred to as the NKCC1 (involved in cellular homeostasis) and the NKCC2 (involved in the re-absorption of Na^+, K^+ and Cl^- found in the kidney and has 6 splice variants). Both isoforms have an approximate mass of 120-160 kDa depending upon the variant (Flatman, 2002).

The regulation of the cotransporter is complex and involves a number of extracellular and intracellular signals. The most simple (and relevant to most transporters), is that the rate of ion movement depends upon the extracellular ion concentration where the rate of transport is decreased at low ion concentrations and saturated at a high ion concentration (Flatman, 2002). Besides ion concentration, the cotransporter is also regulated by protein phosphorylation (Flatman & Creanor, 1999), intracellular Mg^{2+} concentration (increased activity with higher concentrations; needed for kinase and phosphatase activity; Flatman 1988), protein interactions (for example the actin cytoskeleton; Matthews *et al.*, 1994; Matthews *et al.*, 1995), ATP (though phosphorylation not by direct consumption; Flatman, 1991) and intracellular Cl^- ($[Cl^-]_i$) concentration (increased activity associated with a decrease in $[Cl^-]_i$; Flatman 2002; Liedtke & Cole, 2002). Recently, it has also been shown that the activity can be regulated by ERK (Liedtke & Cole, 2002) and interestingly the ERK 1 has been implicated with the recovery in matrix synthesis in chondrocytes (Hopewell & Urban, 2003).

The NKCC is activated by hyper-osmolality and cell shrinkage (Waldegger *et al.*, 1998; O'Neill, 1999; Liedtke & Cole, 2002) although currently the exact mechanism is unknown. The activation of the NKCC initiates an influx of Na^+, K^+ and Cl^- and subsequently the restoration of cell volume due to the influx of osmotically obliged water. As mentioned previously, the cotransporter is activated by Cl^- -sensitive phosphorylation. Currently, the exact mechanism of regulation is unclear although is thought to involve a series of kinases and phosphatases. At least five different consensus sites have been identified, with the phosphorylation of threonine (thr) and serine (ser) residues playing a key role in the regulation of the transporter. As different mechanisms of cotransporter activation (hyper-osmotic, cAMP, thr/ser phosphatases inhibitors and fluoride) gave the same patterns of phosphorylation (Lytle, 1997), this lead to the idea of a one kinase/phosphatases mode of regulation. Currerntly, as a specific kinase inhibitor has not been found that blocks all cotransporter activity (Staurosporine; and genistein are good inhibitors but do not mediated complete inhibition (Lytle, 1997; Flatman & Creanor, 1999) and therefore a multiple kinase model for activation has been suggested (Flatman, 2002).

Another possible regulatory of the NKCC is the cellular F-actin cytoskeleton. It has been shown in T84 epithelia cells that the activation of the NKCC is sensitive to a polymerised F-actin cytoskeleton; with a decrease in activity upon actin stabilisation by phalloidin (Matthews *et al.*, 1994; Matthews *et al.*, 1995; Dandrea *et al.*, 1996). Furthermore, it has been shown that NKCC activity is actually increased upon F-actin depolymerisation (Matthews *et al.*, 1997) and therefore may be regulated by short actin filaments as opposed to the polymerised F-actin cytoskeleton per se.

1.7.2 Na^+/H^+ exchange.

The second membrane transporter that has been shown to be involved in RVI is the Na^+/H^+ exchange coupled to the HCO_3^-/Cl^- exchanger. The Na^+/H^+ exchange is a member of the Sodium Hydrogen Exchanger (NHE) family involved in mediating the electroneutral exchange of one Na^+ ion for one H^+ ion (Aronson, 1985). To date six isoforms (termed NHE1 to NHE6) have been identified, that differ in tissue location and pharmacology with an overlap in form and function (Counillon & Pouyssegur, 2000). The NHE1 is almost ubiquitous expressed (in mammalian tissues) compared to the NHE5 that is predominately found in the brain and NHE3 that is mainly located in the kidney (Counillon & Pouyssegur, 2000). Interestingly, and unlike all the other NHEs, NHE6 is intracellular (although widely expressed) and associated with the mitochondria membrane (Numata *et al.*, 1998). The isoforms have ~45-65 % amino acid sequence homology, sometimes exist as oligomers (Fafournoux *et al.*, 1994) with a hydrophobic N-terminus (10-12 transmembrane regions) and a hydrophilic cytoplasmic C-terminus (Sardet *et al.*, 1990; Counillon & Pouyssegur, 2000). The exchanger is involved in the regulation of cell pH_i, transepithelial Na^+ transport as well as cell volume homeostasis (Hoffman & Simonsen, 1989; Wilkins *et* al., 1996; Counillon & Pouyssegur, 2000).

The six isoforms of the exchanger differ in their regulation and sensitivity to pharmacological agents. The activation and inactivation of all the isoforms of the exchanger are sensitive to changes in intracellular ATP concentration. Cell shrinkage (and consequently an increase in intracellular ATP) up-regulates activity (Wilkins *et* al., 1995; Counillon & Pouyssegur, 2000) whereas cell swelling (and consequently an apparent decrease in ATP) decreases activity (Counillon & Pouyssegur, 2000).

Differences in response to the same stimuli are shown by cAMP and protein kinase A (PKA) sensitivity; NHE 3 activity is down regulated by cAMP and (PKA) whereas NHE 1 is unaffected (Counillon & Pouyssegur, 2000). NHE activity can also be influenced by the F-actin cytoskeleton where in primary rat astrocytes it has been shown that NHE1 activity is up-regulated in response to hyper-osmolality) via the activation of calmodulin-dependent MLCK (Shrode *et al.*,, 1995; Shrode *et al.*, 1997).

It has also been shown that the Na^+/H^+ exchanger is comprised of subunits and that these domains confer specificity to the molecule (Fafournoux *et al.,* 1994). Using dimmers made of NHE1 subunits and in the cell line PS120, it was found that the exchanger exists as a homodimer in the cell membrane and as NHE1 did not dimerise with NHE3 it was suggested that each has specific coding that identifies the type of exchanger (Fafournoux *et al.,* 1994). Furthermore, each monomer functioned independently within the dimmer but it was suggested by the author that the formation of the homodimer may still be required for activity (Fafournoux *et al.,* 1994).

With respect to volume regulation, the activity of the Na^+/H^+ exchanger is 'coupled' to the activity of the Cl^-/HCO_3^- exchanger with a resultant efflux of H^+ and HCO_3^-. Overall, this leads to the influx of water and the recovery of cell volume in response to an increase in extracellular osmolality. The extruded H^+ and HCO_3^- combine (mediated by carbonic anhydrase) to form CO_2, which then re-enters the cell to regenerate H^+ and HCO_3^- (Sibley *et al.*, 2002). For example, in EAT cells, it has been shown that in the presence of HCO_3^- medium ~35% of RVI is mediated by the NHE (Hoffmann, 1997)

and in rat pancreatic beta-cells RVI is also mediated by the NHE in the presence of HCO_3^- (Miley *et al.*, 1998).

1.7.3 Na^+-K^+ pump.

The last membrane transporter shown to be implicated in the RVI response is the ATP dependent, membrane bound Na^+-K^+ pump (Waldegger *et al.*, 1998; O'Neill 1999). It is involved the maintenance the cellular Na^+ - K^+ ion gradient and volume homeostasis by the stoichiometric exchange of $2K^+$ ions for $3Na^+$ ions (Tsong & Chang, 2003). The Na^+-K^+ pump is comprised of two domains (termed α and β) each of which have different isoforms (Tsong & Chang, 2003). With respect to RVI, the net Na^+ influx mediated by either the NHE, NKCC (or in some cases the Na^+/HCO_3^-) stimulates the activity of the Na^+-K^+ pump that subsequently drives the substitution of Na^+ for K^+ and the maintenance of $[K^+]_i$ required for optimal metabolism and the restoration of cell volume (Horowitz & Lau Y-T, 1988; Hoffmann, 1992; Hoffmann & Mills, 1999).

1.7.4 Na^+/HCO_3^- symport.

The Na^+/HCO_3^- exchange (HBC) is found in several mammalian cell types although interestingly the direction and the stoichiometry of the channel is dependent upon the type of tissue (Soleimani & Burnham, 2000). For example, in the kidney the symport mediates the extrusion of HCO_3^- (termed 'acid loader') in a ratio of 3:1 (HCO_3^-: Na^+; Boron & Boulpaep, 1989; Aronson *et al.*, 1991; Soleimani *et al.*, 1994). Conversely, in heart cells the symport mediates the entry of HCO_3^- (termed 'alkaline loader') in a ratio of 2:1 (Lagadicgossmann *et al.*, 1992). The role for the symport in RVI is less

documented although in osteosarcoma cells (UMR-106-01), it has been shown that RVI is insensitive to amiloride although inhibited by the removal of extracellular Na^+ or by the addition of DIDS - indicative of symport activity (Star *et al.*, 1992).

1.7.5 RVI in articular chondrocytes.

Having considered the membrane transporters involved in RVI, reviewing their expression in articular chondrocytes would help in an understanding of chondrocyte volume regulation. The presence of a bumetanide-sensitive ^{86}Rb influx has been shown in isolated bovine articular chondrocytes (Hall *et al.*, 1996a, 1996b), where isolated chondrocytes were placed into a hyper-osmotic DMEM and the influx of ^{86}Rb measured. It was found that the ^{86}Rb influx was stimulated by hypertonicity and inhibited in the presence of bumetanide. Furthermore, immunohistochemistry has shown that the NKCC protein is present in human articular chondrocytes and interestingly with no difference between 'non-degenerate' and 'degenerate' cartilage (Trujillo *et al.*, 1999). These data would suggest that chondrocytes have a functional NKCC co-transporter and that it is activated in response to an increase in extracellular osmolality with no change during the progression of osteoarthritis.

The presence of the Na^+/H^+ exchanger has been shown in human articular chondrocytes by 2D gel electrophoresis. Using specific antibodies for the Na^+/H^+ exchanger it was found that the NHE 1, NHE 2 and NHE 3 isoforms are expressed and it was shown that there was no change in expression associated with the progression of osteoarthritis (Trujillo *et al.*, 1999). Subsequent work on bovine articular chondrocytes have shown that the NHE can be stimulated by both hydrostatic pressure and changes in

extracellular osmolality (Wilkins *et al.*, 1996; Browning *et al.*, 1999; Wilkins *et al.*, 2000a; Yamazaki *et al.*, 2000). The Na^+/H^+ exchange is also activated by a change in pH with pH_i regulation in response to an acid load is inhibited by amiloride (Browning *et al.*, 1999; O'Neill *et al.*, 2002).

In bovine articular chondrocytes, the presence of the Na^+-K^+ pump has been confirmed by 2D gel electrophoresis and *in situ* immunofluorescence. It was found that there was the expression of 2 alpha isoforms (α 1 & α 3) and 2 beta isoforms (β 1 and β 2) and that the expression of the pump was sensitive to extracellular sodium concentration (Mobasheri *et al.*, 1996; Mobasheri *et al.*, 1997).

Despite the expression of all three major membrane transporters known to mediate RVI, there has been very little work on RVI in chondrocytes. *In situ* studies on bovine articular chondrocytes, where cartilage explants were incubated with the fluorescent intracellular dye CMFDA and the chondrocytes visualised by confocal microscopy have shown that in response to a modified form of the post RVD-RVI protocol[2], chondrocytes underwent strong 'post RVD-RVI' despite little evidence of RVD (Errington *et al.*, 1997). In porcine articular chondrocytes the capacity for slow RVI has also been shown as in response to a increase in extracellular osmolality and interestingly the RVI was not mediated by the hyper-osmotic induced rise in $[Ca^{2+}]_i$ (Erickson *et al.*, 2001).

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[2] The standard post RVD-RVI protocol involes applying a hypo-osmotic challenge to the cells for a set period and then returning the cells to a solution of the original osmolality. By returning the cells to the original solution, this is in effect a hyper-osmotic challenge as the original solution has a higher osmolality than that of the hypo-osmotic one. In this example, the modified protocol involved having the cells in an original media (280mOsm.kg H_2O^{-1}) and then a hypo-osmotic challenge applied (saline changed to 140mOsm.kg H_2O^{-1}). After a set period in the hypo-osmotic saline, and instead of returning the cells to the original saline, a saline of 380mOsm.kg H_2O^{-1} was applied.

In summary, it has been shown that articular chondrocytes are able to volume-regulate in response to an increase in extracellular osmolality though the mechanism remains unclear. It would appear that chondrocytes have a functional Na-K-2Cl cotransporter as well as various isoforms of the Na^+/H^+ exchange and the Na^+-K^+ pump.

1.8.0 The Role of $[Ca^{2+}]_i$ and Volume Regulation.

In many cell types a decrease in extracellular osmolality results in a transient rise in $[Ca^{2+}]_i$ as a result of calcium influx and in some cells a release from intracellular stores (Tinel *et al.*, 2000; Weskamp *et al.*, 2000a). It has often been observed (for example in kidney, EAT and celebellar cells) that the rise in $[Ca^{2+}]_i$ is bi-phasic where the initial rise is due to a release from intracellular stores and the sustained elevation is a result an influx across the cell membrane (Oconnor & Kimelberg, 1993; Tinel *et al.*, 1994; Tinel *et al.*, 2000). Conversely, in response to an increase in extracellular osmolality very few cell-types respond with a rise in $[Ca^{2+}]_i$ (Hoffman & Dunnham, 1995; Hoffman & Mills, 1999). The literature on the role of $[Ca^{2+}]_i$ in mediating volume-regulation is complex and it would appear that the role is dependent upon the cell type being studied.

Numerous pathways, including the voltage activated calcium channels (VACCs) and the stretch sensitive channels (SACs; Wright *et al.*, 1996; Tinel *et* al., 2000; Yellowley *et al.*, 2002) mediate calcium influx. A voltage sensitive calcium influx has been observed in many cell types including: astrocytes, chondrocytes, kidney cells and osteocytes (Oconnor & Kimelberg, 1993; Miyauchi *et al.*, 2000; Tinel *et al.*, 2000). In rat and chicken osteocytes, a decrease in osmolality results in an influx of calcium mediated via a gadolinium sensitive L-type calcium channel (Miyauchi *et al.*, 1996; Miyauchi *et al.*, 2000). Inhibiting the rise in $[Ca^{2+}]_i$ significantly attenuates the RVD response thus implying a role for calcium in volume regulation (Miyauchi *et al.*, 2000). Conversely in rat cerebellar granule neurons abolition of the hypo-osmotic rise in $[Ca^{2+}]_i$ had no effect on the capacity for RVD suggesting that in this cell-type calcium is unlikely a mediator of the response (Moran *et al.*, 1997; Morales-Mulia *et al.*, 1998).

1.8.1 Calcium Signalling and Volume Regulation in Chondrocytes.

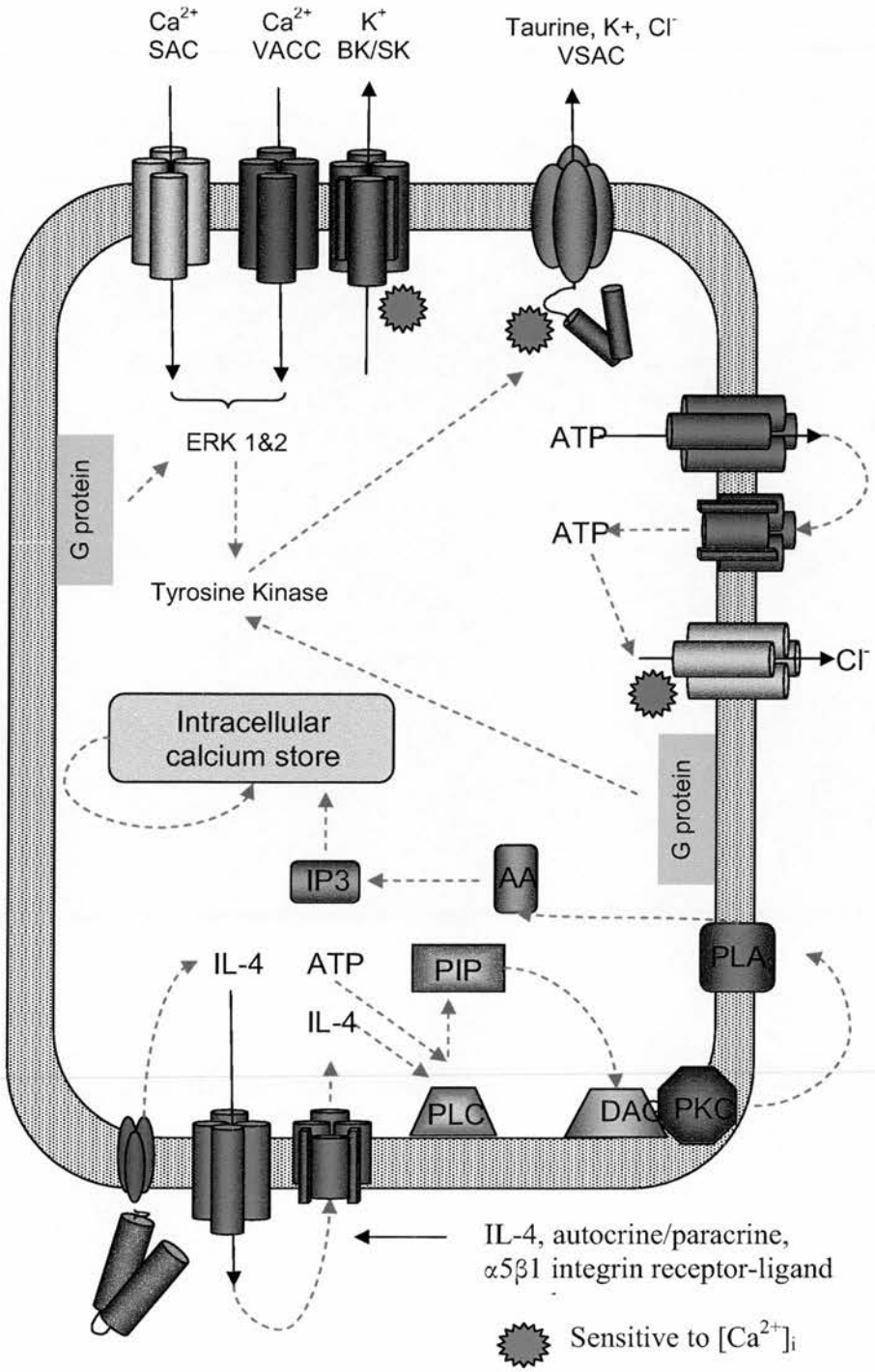
There has been little work on the mechanism(s) of volume-regulation in articular chondrocytes. It has been shown that these cells have the capacity for volume regulation yet the channels that mediate the response have not been identified. As mentioned in section 1.8.0, changes in $[Ca^{2+}]_i$ have been linked to volume-regulation in other cell types (Miyauchi *et al.*, 2000) and in chondrocytes changes in $[Ca^{2+}]_i$ have been observed in response to changes in osmolality (Kerrigan & Hall, 2000). Furthermore, it is currently unclear how chondrocytes 'sense' a change in osmolality and it is possible that the response is mediated via one of the mechanisms discussed in section 1.5.0

In bovine articular chondrocytes, a decrease in osmolality results in an increase in $[Ca^{2+}]_i$ as a result of an influx across the cell membrane (Kerrigan & Hall, 2000; Yellowley *et al.*, 2000, 2001, 2002). The response was transient and always occurred after a delay of at least 60 seconds where it was found that the percentage of cells responding was inversely proportional to the osmotic challenge. Pharmacological data has shown that the rise is attributed to both a calcium influx and a release from intracellular stores. The addition of gadolinium chloride (non-selective inhibitor of stretch sensitive ion channels; (Caldwell *et al.*, 1998), the removal of $[Ca^{2+}]_o$ or addition of thapsigargin (an inhibitor of the intracellular ATP-dependent calcium pump and results in Ca^{2+} release from stores) all attenuated the $[Ca^{2+}]_i$ response but not always RVD (Kerrigan & Hall, 2000; Yellowley *et al.*, 2000, 2001, 2002).

Unlike most cell types studied to date, an increase in extracellular osmolality also initiates a rise in $[Ca^{2+}]_i$ in both bovine and porcine articular chondrocytes (Erickson *et al.*, 2001; section 4.2.4). In porcine articular chondrocytes the $[Ca^{2+}]_i$ rise is a result of a gadolinium sensitive influx from the extracellular environment as well as a release from IP_3 sensitive intracellular stores, potentially mediated by G-proteins and PLC (Erickson *et al.*, 2001). Interestingly, the inhibition of the hyper-osmotic induced rise in $[Ca^{2+}]_i$ resulted in further cell shrinkage with an associated increase in the percentage of volume recovery via RVI. The reason for this was not know although it was postulated by the author that this may be a result of cell damage and consequently an artefact (Erickson *et al.*, 2001). Recently, work has shown that chondrocytes of the invertebral disk (more specifically chondrocytes from the annulus fibrosus, and transition zones) also respond to an increase in extracellular osmolality with a rise in $[Ca^{2+}]_i$. The rise is a result of an influx from the extracellular environment and mediated by the polymerisation state of the F-actin cytoskeleton (Pritchard *et al.*, 2002).

To summarise, articular chondrocytes like most cells respond to a decrease in extracellular osmolality with a rise in $[Ca^{2+}]_i$. The rise would appear to involve an influx from the extracellular environment, mediated via a stretch sensitive anion channel. Calcium release from intracellular stores (IP_3 and potentially arachidonic acid sensitive) also appears to be involved, although the exact mechanism is currently unclear. These changes in $[Ca^{2+}]_i$ may be involved in the volume regulatory response or in regulating matrix synthesis, although as observed in many other cell types, the rise may just be a result of an epiphenomenon (Moran *et al.*, 1997; Morales-Mulia *et al.*, 1998).

Due to the complexity of the chondrocytes extracellular environment, as well as the potentiall pathways that may be involved in sensing changes in extracellular osmolality, figure 1.5 illustrates some of the signalling and volume-regulatory mechanisms shown to be present in chondrocytes that may be involved in the volume-regulatory response. As discussed previously (section 1.5.0), there is a degree of overlap between all these pathways and it is possible that there is no single 'volume-regulatory sensing mechanism' and that pathways are interlinked, resulting in a complex, signalling/sensory network.



AA	Arachidonic acid	PKC	Protein Kinase C
BK/SK	Large conductance/Small conductance K^+ channel	PLA_2	Phospholipase 2
		PLC	Phospholipase C
DAG	Diacylglycerol	SAC	Stretch activated Channel
ERK	Extracellular Signal-Regulated Protein Kinase	VACC	Voltage Activated Calcium Channel
IP_3	Inositol-(1,4,5)-triphosphate	VSAC	Volume Sensitive Anion Channel
IL-4	Interleukin 4		

Figure 1. 5. Potential signalling and volume regulatory pathways in chondrocytes.

In chondrocytes, numerous signalling pathways have been described, and many membrane transporters implicated in chondrocyte mechanosensation. In many of the responses, changes to $[Ca^{2+}]_i$ are often observed and in some cases (for example in response to pressure-induced strain) implicated in the response. The diagram on the opposite page is a summary of some of the responses. The diagram shows an 'a typical' styalised chondrocyte with the plasma membrane shown in grey. Channels and transporters involved in chondrocyte mechanotrnsduction are labelled and the key given at the bottom of the page. Signalling events are represented by dashed blue lines, and the flow of ions/molecules shown as black full lines. A red 'callout'symbol represents pathways that are activated by calcium. A chondrocyte may perceive a change in volume as membrane stretch or strain and this leads to the activation of the IL-4 and Gd^{3+} sensitive Ca^{2+} influx. This may then lead to the release of calcium from intracellular stores and the activation of calcium sensitive K^+ and Cl^- efflux). A decrease in osmolality results in the activation of the chondrocyte 'osmolyte channel' and RVD. G-protein and ERK activation have also been shown to be involved in response to a decrease in osmolality and may play a role either in the RVD response or in the maintenance of matrix synthesis. For references, please refer to text.

1.9.0 The Actin Cytoskeleton.

The actin cytoskeleton is a dynamic, intracellular structure involved in a wide range of cellular functions. It is comprised of monomeric G-actin subunits that when polymerised (regulated by subsidiary binding proteins including gelsolin, profiling and cofilin; (Jakab *et al.*, 2002) form the polymerised F-actin cytoskeleton. In chondrocytes, it has previously been shown that changes in the actin cytoskeleton influence the phenotype of the cell and consequently the type of collagen synthesised (Benya *et* al., 1978; Benya *et al.*, 1988; Pirttiniemi & Kantomaa, 1998; Loty *et al.*, 2000). As well as being directly involved in phenotypic regulation, the actin cytoskeleton has also been shown to be an important mediator in mechano-transduction (Lee *et al.*, 2000; Millward-Sadler *et al.*, 2000a). Therefore, as the actin cytoskeleton is crucial in chondrocyte function, the role of the actin cytoskeleton in volume regulation will be reviewed.

1.9.1 Study of the Actin Cytoskeleton

The study of actin cytoskeleton has been greatly enhanced by the use of the Cytochalasin fungal toxins, and the Latrunculin marine red sponge toxins. Within these two types of actin depolymerising toxins, sub-types are found, each of which influence the actin cytoskeleton (or the cell) in slightly different ways. These different agents and their effects are outlined in Table 1.3. Due to the different modes of action, or the effects on the cell, when reviewing studies on the actin cytoskeleton, knowing the type of toxin used is important in the inference of the results.

Besides using toxins that disrupt the actin cytoskeleton, compounds that stabilise and prevent actin disassembly are also widely used. The most common of these is Phalloidin, a bicyclic heptapeptide isolated from the fungi *Amanita phalloides* (common name: Green Deathcap mushroom; Wieland *et al.*, 1983). It binds specifically to polymerised (F) actin and prevents the disassembly into monomeric (G) actin (Small *et al.*, 1988). Phalloidin is often tagged with fluorophores, for example FITC or TRITC and is used as a method to visualise the actin cytoskeleton (Harkin & Hay, 1996; Mair *et al.*, 1998; Kotikova *et al.*, 2001).

Type	Subtype	Influences on the actin cytoskeleton	Other cellular influences	Reference
Cytochalasin	A	Inhibits actin polymerisation.	Interferes with microtubule assembly.	Lingham *et al.*, 1992; Torralba *et al.*, 1998
Cytochalasin	B	Blocks monomer addition. Prevents the formation of contractile filaments.	Inhibits division, cell movement and induces nuclear extrusion. Inhibits glucose transport.	Tanaka et al., 1994; Theodoropoulos et al., 1994
Cytochalasin	D	x10 potency of B. Blocks monomer addition.	Does not inhibit monosaccharide transport. Does inhibit low conductance K^+ channels.	Aszalos *et al.*, 1994; Wang *et al.*, 1994; Sasaki *et al.*, 1995
Cytochalasin	E	Inhibits actin polymerisation stimulated by F-actin.	Does not inhibit glucose transport.	Mookerjee & Jung, 1984
Latrunculin	A	Binds to monomeric (G) actin disrupting microfilament organisation.	Inhibitor of microfilament-mediated processes in sperm, eggs, and embryos.	Spector & Shochet, 1983; Schatten *et al.*, 1986; Ayscough *et al.*, 1997
Latrunculin	B	Inhibits polymerisation and disrupts microfilament organisation. x10-x100 more potent than cytochalasins.	N/A	Spector & Shochet, 1983; Blasberger et al., 1989

Table 1. 3. Inhibitors of the actin cytoskeleton

The study of the actin cytoskeleton has been greatly enhanced by the use of specific fungal Cytochalasin toxins and marine sponge Latrunculin toxins. The table details mode of action and any adverse cellular effects.

1.9.2 Organisation of the Actin Cytoskeleton in Chondrocytes.

As previously mentioned, the organisation of the actin cytoskeleton is closely associated with the phenotype of the chondrocyte. Consequently, as it has previously been shown that the expressed phenotype can influence the type of collagen synthesised (and therefore affect the viability of the cartilage matrix), the organisation of the actin cytoskeleton has become an intense area of study. In chondrocytes, the differentiated and the fibroblastic phenotypes (and how they can be influenced) are well studied and the organisation of the actin cytoskeleton defined.

1.9.2.1 Differentiated Phenotype.

The differentiated phenotype (as found *in-situ*) is confirmed by the expression of collagen type II and aggrecan (Benya & Shaffer, 1982; Aigner *et al.*, 1995; Toolan *et al.*, 1996). In full depth organ cultures, it has been shown (using CLSM) that polymerised (F) actin is located cortically, just below the plasma membrane with little staining though the cell cytosol (Durrant *et al.*, 1999; Dumas *et al.*, 2000; Langelier *et al.*, 2000). Comparisons by western blot analysis using a mouse anti-actin antibody between the three main cartilage zones (superficial, mid and deep), revealed a greater actin label intensity in the superficial zone (Langelier *et al.*, 2000) with an equal distribution between the mid and deep zones. It was postulated that this higher level of actin may relate to an increased level of remodelling although interestingly, it has previously been shown that mechanical stimulation on cartilage explants does not increase the level of (F) actin (Langelier *et al.*, 2000).

As the study of the chondrocytes *in situ* is complex due to the nature of the extracellular matrix, chondrocytes are often isolated and cultured in 3D (including alginate, agarose, and collagen sponges) as the differentiated phenotype is maintained (as shown by collagen II and aggrecan synthesis; (Benya & Shaffer, 1982; Benya *et al.*, 1988; Hauselmann *et al.*, 1992; Hauselmann *et al.*, 1994; Frenkel *et al.*, 1996; Hauselmann *et al.*, 1996; Chubinskaya *et al.*, 2001). As observed *in situ*, polymerised (F) actin was located cortically with little staining through the cell cytosol although this organisation was not clearly present until 3-7 days after the initial seeding (Dumas *et al.*, 2000; Idowu *et al.*, 2000; Guilak *et al.*, 2002). The organisation of the cytoskeleton was correlated to the synthesis of hydroxyproline and GAG and it was found that an increased level of synthesis was associated with an organised cytoskeleton (Idowu *et al.*, 2000).

1.9.2.2 De-differentiated (Fibroblastic) Phenotype.

As a result of monolayer culture, chondrocytes de-differentiate into a fibroblastic form as shown by the switch in synthesis of collagen type II to collagen type I, the loss of collagen IX & XI and the expression of cathepsin B (Benya & Shaffer, 1981; Benya *et al.*, 1981; Benya & Shaffer, 1982; Baici *et al.*, 1988; Liu *et al.*, 1998a; Zwicky & Baici, 2000a). The actin cytoskeleton of de-differentiated chondrocytes is more pronounced when compared to *in situ* or 3D cultured chondrocytes, the sub-cortical arrangement is lost and actin cables that span the whole cell are clearly visible (Mallein-Gerin *et al.*, 1991; Idowu *et al.*, 2000; Loty *et al.*, 2000; Zwicky & Baici, 2000a). Interestingly, it has previously been shown there are some similarities between de-differentiated chondrocytes and those present in OA cartilage as both produce collagen type I

(although this is under review; Gebhard *et al.*, 2003), and Cathepsin B (Baici *et al.*, 1995a; Baici *et al.*, 1995b).

1.9.3 The actin cytoskeleton and the chondrocytic phenotype.

It is clear that the organisation of the actin cytoskeleton is related to the expressed phenotype, and that changes in the culture conditions influence its organisation (Benya *et al.*, 1981). As the type of collagen synthesised by chondrocytes is important in maintaining a viable extracellular matrix, the regulators of collagen synthesis and the collagen network (and thus the chondrocytic phenotype) have become an intense area of study (Eyre & Wu, 1995; Muir, 1995). As shown in Table 1.4, in mammals, five phenotypes have been classified, each responsible for the synthesis of a particular set of collagens.

In mature healthy cartilage, chondrocytes primarily synthesise type II collagen. Conversely (as shown by collagen gene analysis), it has been found in mid to late osteoarthritis (OA) there was a switch in the type of collagen synthesised to Types IIA (foetal), Type X (hypertrophic) and types I & III (de-differentiated; Aigner *et al.*, 1999; Aigner & McKenna, 2002). This led to what has now been described as the '*osteoarthritic phenotype*' as ordinarily each set of sub-type collagens are usually phenotypically exclusive. Worthy of note are quantitative PCR studies performed by Gebhard *et al.*, (2003) comparing chondrocytes from 'normal' and degenerate cartilage. It was shown (with the exception of collagen type X) that the ratios between the gene expression of the collagens were not different, and therefore during OA there may not be a change in the expressed phenotype (for example as seen in the chondrocytic to

fibroblastic phenotypes). It was suggested that there may just be an alteration in the amount of the different collagens synthesised whilst maintaining the healthy chondrocytic ratio.

Phenotype	Collagen	Reference
Foetal	Type IIA	Hayes et al., 2001
Differentiated (mature)	Type II, III, IX, XI	Benya & Shaffer, 1982; Benya et al., 1988; Hauselmann et al., 1992; Hauselmann et al., 1994; Frenkel et al., 1996; Hauselmann et al., 1996; Chubinskaya et al., 2001
Fibroblastic	Type I, III	Benya & Shaffer, 1981; Benya et al., 1981; Benya & Shaffer, 1982; Baici et al., 1988; Liu et al., 1998a Zwicky & Baici, 2000a
Hypertrophic	Type X	Ainger & McKenna 2002
Osteoarthritic	Type IIA, III, X, VI	Aigner et al., 1995; Soder et al., 2002; Gebhard et al., 2003

Table 1.4. The Chondrocytic phenotype.

The above table summarises the different chondrocytic phenotypes in relation to the types of collagen synthesised.

Using different drugs that influence actin polymerisation, the chondrocytic phenotype and consequently the type of collagen synthesised can be altered (Pirttiniemi & Kantomaa, 1998; Loty *et al.*, 2000). In 2D monolayer culture, chondrocytes de-differentiate to a fibroblastic form with a loss of the differentiated rounded cell shape in conjunction with the formation of stress fibres. There is also a switch in the type of collagen synthesised principally from type II to type I (Benya & Shaffer, 1981; Benya *et al.*, 1981; Benya & Shaffer, 1982; Baici *et al.*, 1988; Idowu *et al.*, 2000). Interestingly, if the chondrocytes are then incubated with a drug that depolymerises the actin cytoskeleton (for example Cytochalasin B or D), the phenotype has been shown to revert back to the differentiated form with the type of collagen synthesised switched back to type II (Mallein-Gerin *et* al., 1991; Pirttiniemi & Kantomaa 1998; Sautier & Forrest, 2000). This therefore suggests that it is possibly the change in cell shape or the formation of actin stress fibrils that mediate the changes in the type of collagen synthesised.

Unlike 2D culture, 3D culturing techniques (including alginate, agarose, and collagen sponges) have been shown to maintain the chondrocytic phenotype and indeed revert a fibroblastic-like chondrocyte to the differentiated phenotype (Benya & Shaffer, 1982; Hauselmann *et al.*, 1992; Hauselmann *et al.*, 1994; Frenkel *et al.*, 1996; Hauselmann *et al.*, 1996; Chubinskaya *et al.*, 2001). Recent work using two 'novel' indicators of the differentiated phenotype (Collagen type IX and Cartilage Oligomeric Matrix Protein; COMP) it was shown that collagen type II mRNA can be detected for up to 9 weeks in 2D culture and is not lost until week 16. Furthermore, the time for phenotypic recovery was directly proportional to the time in monolayer culture and the initial mRNA levels

were never recovered. Conversely for COMP, down regulation was observed after 2 weeks in monolayer, but the recovery in expression was complete when placed into alginate after only 4 weeks with comparable expression levels (Zaucke *et al.*, 2001). These timings are significantly important when using 2D monolayer cultures to model *in situ* chondrocytes, as knowing when the phenotype changes should allow for the development of more accurate cell models.

The exact mechanism by which the chondrocyte actin cytoskeleton influences the type of collagen synthesised still remains unknown. It has been suggested that the switch in synthesis is unlikely to be mediated solely by a change in cell shape as chondrocyte monolayers can be stimulated to produce type II collagen in the presence agents of that depolymerise the f-actin cytoskeleton without a further change in morphology (Benya *et al.*, 1988; Mallein-Gerin *et al.*, 1991; Loty *et al.*, 2000). Furthermore, as 3D cultured chondrocytes have been shown to have an elaborate cortical actin cytoskeletal network, it has been suggested that actin polymerisation as such is not necessarily the signal and that the formation of stress fibres may be (Idowu *et al.*, 2000). It has been commented by Loty *et al* (2000) that the cytochalasins may influence the nuclear cytoskeleton and consequently gene expression although this is still to be proven.

In summary, the chondrocyte actin cytoskeleton and the expressed phenotype are closely linked. Changes in the phenotype as a result of monolayer culture, induce a significant re-organisation or the actin cytoskeleton resulting in the formation of stress fibres and a change in chondrocyte matrix synthesis. Phenotypic recovery can be mediated by placing fibroblastic chondrocytes in to a 3D culture system or by disruption

of the actin cytoskeleton. Currently, the signalling pathways for these changes is unclear although it is thought that the changes in cell morphology are secondary to the re-organisation of the actin cytoskeleton and may involve changes in the nuclear cytoskeleton.

1.9.4 The actin cytoskeleton and volume regulation.

It is well documented, that changes in extracellular osmolality result in a re-organisation of the actin microfilaments in many cell types (Moustakas *et al.*, 1998; Hoffmann, 2000). In most cases, a decrease in extracellular osmolality results in a loss of the polymerised F-actin and the formation of small sub-membranous F-actin aggregations as seen in porcine articular chondrocytes and EAT cells (Moustakas *et al.*, 1998; Pedersen *et al.*, 1999; Pedersen *et al.*, 2001; Guilak *et al.*, 2002; Erickson *et al.*, 2003). Conversely, an increase in extracellular osmolality generally results in an increase in cellular F-actin for example as seen in rat peritoneal mast cells and in concordance with data from human neutrophils (Rizoli *et al.*, 2000; Di Ciano *et al.*, 2002; Guilak *et al.*, 2002; Koffer *et al.*, 2002; Lewis *et al.*, 2002). Interestingly, an increase in extracellular osmolality does not increase cellular F-actin in freshly isolated procine articular chondrocytes (Guilak *et al.*, 2002).

The involvement of the actin cytoskeleton in volume regulation has been the subject of intense research and the consensus would appear that the involvement depends upon the cell type in question. In Ehrlich ascites tumour cells (EAT cells), RVD is mediated by the activation of Cl^- and K^+ channels whereas RVI is mediated by the NKCC

cotransporter (with NHE activity resulting in an intracellular alkalisation; (Hoffmann & Pedersen, 1998; Hoffmann, 2000; Niemeyer *et al.*, 2000a, 2000b; Hoffmann & Hougaard, 2001; Pedersen *et al.*, 2001). As previously mentioned, in these cells (EAT cells), a decrease in extracellular osmolality results in a loss of the cortical F-actin structure where conversely an increase in osmolality actually increases F-actin cortical content. A pre-incubation with cytochalasin B inhibited both the RVD and RVI response but interestingly, incubation with cytochalasin D (more specific for actin that cytochalasin B; see Table 1.3) had no effect on the response (Cornet *et al.*, 1993; Pedersen *et al.*, 1999; Hoffmann, 2000; Pedersen *et al.*, 2001). In contrast, it has been found in HL-60 cells and human peripheral blood neutrophils, that RVD was completely unaffected by both cytochalasin B and D treatment although interestingly RVD in Jurkat lymphoma cells was completely inhibited (Downey *et al.*, 1992; Downey *et al.*, 1995) Later work showed that the F-actin cytoskeleton was only depolymerised in Jurkat lymphoma cells by cytochalasin treatment and therefore the possibility that the F-actin cytoskeleton was involved in RVD remains (Downey *et al.*, 1995),

One potential way in which the actin cytoskeleton can influence the volume regulatory response is by the direct regulation of the channel(s) used to mediate the recovery in volume. In many cell types, the NKCC cotransporter mediates RVI (O'Neill, 1999) (Pedersen *et al.*, 2001; Flatman, 2002). It has been shown that the stabilisation of the actin cytoskeleton decreases cAMP stimulation of the cotransporter where conversely, actin depolymerisation (as a result cytochalasin D treatment) stimulates transporter activity (Matthews *et al.*, 1994; Matthews *et al.*, 1995; Dandrea *et al.*, 1996). The transporter is also activated by changes in extracellular osmolality where for example,

shrinkage-induced activated observed in T84 cells (human colonic carcinoma cells; Winsor *et al.*, 1992), is not inhibited by phalloidin but swelling-induced activation in both T84 cells and in EAT cells, is inhibited by cytochalasin D (Matthews *et al.*, 1998). Based on these observations, it has therefore been suggested that an intact/organised F-actin cytoskeleton is required to mediate NKCC activation and not remodelling in response to a change in osmolality (Matthews *et al.*, 1998). Further implicating the actin cytoskeleton in the regulation of the NKCC is the fact that swelling-induced NKCC activation is inhibited by the Myosin Light Chain Kinase (MLCK) inhibitor ML-7 as observed in EAT, eel intestinal epithelium and T84 cells (Lionetto *et al.*, 2002).

Alternatively, the F-actin cytoskeleton could form part of the signalling pathways required to initiate a volume regulatory response. For example, in Intestine 407 cells, a decrease in extracellular osmolality results in an increase (and re-organisation) of the cellular F-actin cytoskeleton and subsequent RVD (Tilly *et al.*, 1993; Tilly *et al.*, 1994; Tilly *et al.*, 1996). It was suggested that the osmotic challenge and the subsequent re-organisation of the F-actin cytoskeleton induced $p125^{FAK}$ activation (a tyrosine kinase associated with the cytoskeleton and focal adhesions), and this in conjunction with $p21^{rho}$ are mediators of the RVD response. Furthermore, inhibitors of these F-actin cytoskeletal modulators (including *Clostridium botulinum* C3 Exoenzyme) where shown to inhibit the Cl^- efflux and consequently RVD.

It is clear that the actin cytoskeleton can influence mediators of both the RVD and RVI response either by direct action on the channel/transporter involved, or by a role in the signalling events leading to the onset of the response. It has also been suggested that a

polymerised actin cytoskeleton may be necessary for the translocation of volume regulatory transports to the plasma membrane from the cellular pool (Hoffmann & Mills, 1999; Pedersen *et al.*, 2001). In serum-deprived Caco-2 cells, the activity of the NHE transport is generally low as vesicle cycling is prevented due to the serum-induced de-polymerisation of the actin cytoskeleton (Watson *et al.*, 1991). Conversely, a pre-treatment with cytochalasin (thus depolymerising the F-actin cytoskeleton) maintained NHE activity, as endocytosis is dependent upon an intact actin cytoskeleton and therefore the NHE transporter proteins were left at the plasma membrane (Watson *et al.*, 1991; Watson *et al.*, 1992; Watson, 1993). Subsequent work on the NHE3 has shown that its activity is in part regulated by the number of transporter proteins expressed on the cell membrane (either by endocytosis and or exocytosis) and thus implicating the actin cytoskeleton in its regulation (Szaszi *et al.*, 2000).

As well as influencing the channels/transporters that mediate volume regulation, the actin cytoskeleton could also form part of the 'volume sensor' required to mediate the volume regulatory response. One possibility is through cellular integrins, found on the cell membrane and closely linked to the actin cytoskeleton (Jakab *et al.*, 2002; Hoffmann & Dunham, 1995; Hoffmann, 2000). In chondrocytes, it has been shown that mechanical stimulation (as a change in volume could be perceived as a mechanical stimulus; Low & Taylor, 1998) in response to pressure-induced strain is mediated by an IL-4, autocrine/paracrine, $\alpha 5\beta 1$ integrin receptor-ligand complex. This in turn results in cell hyperpolarisation mediated by a stretch-induced, gadolinium-sensitive low-conductance, apamin-sensitive, Ca^{2+}- activated K^+ channel (Grandolfo *et al.*, 1990; Grandolfo *et al.*, 1992; Wright *et al.*, 1996; Millward-Sadler *et al.*, 1999; Salter *et al.*,

2000). Conversely, in human neutrophils, the cross linking of β2 integrins using specific antibodies had no effect on the capacity for RVD although did induce actin polymerisation (Downey *et al.*, 1995). Therefore, as previously mentioned, the response appears to be closely related to the cell type studied.

In summary, the chondrocyte phenotype is closely linked to the organisation of the actin cytoskeleton and that actin polymerisation influences the type of collagen synthesised. Changes in cell volume have been shown to influence the organisation of the actin cytoskeleton and these changes may form part of the volume-regulatory response. The actin cytoskeleton may influence the capacity for volume regulation by direct action on a channel or transporter, play a role in the signalling cascade or form part of the volume sensor. In chondrocytes, it is known that both increases and decreases in extracellular osmolality influence the organisation of the actin cytoskeleton although the effects on the capacity for volume regulation are still to be elucidated.

1.10.0 Osteoarthritis

As mentioned previously (see section 1.1.1), articular cartilage protects the underlying bone from potential damage as a result of joint articulation. The degeneration of articular cartilage (either by alterations in structure or composition; (Buckwalter & Mankin, 1997b) ultimately leads to a loss of function and consequently osteoarthritis (OA). Osteoarthritis (or more correctly termed osteoarthrosis as the suffix ***itis*** denotes inflammation and this is not necessarily an early event or a major symptom; Buckwalter & Mankin, 1997b) is clinically referred to as a syndrome, comprising of synovial joint changes including: (1) attempted cartilage repair, (2) subchondral bone cyst formation and (3) sclerosis of subchondral bone. These result in visible symptoms that may include joint swelling, loss of motion and joint deformity (Fig. 1.6).

In the UK, it has been estimated that 4.4 million people have x-ray evidence of moderate to severe OA in their hands, 550,000 have moderate to severe OA in their knees and 210,000 have moderate to serve OA in their hips (figures from 1999; McCormick *et al.*, 1995). Taking these totals together, it was approximated that in 1999-2000, 3.197 billion pounds were lost due to loss of production (i.e. lost working hours) costing the NHS in excess of 327 million pounds (including related diseases) in prescriptions alone (McCormick *et al.*, 1995).

The aetiology of OA is often divided into two types, primary (sometimes referred to as idiopathic) and secondary (Stockwell, 1991). Primary OA is the result of an unknown influence and is more common whereas in secondary OA the cause is easily identifiable.

Multiple factors have been associated with the onset of secondary OA including trauma (acute or dismissive, joint or ligamental), OA from Rheumatoid Arthritis (RA), specific area-related metabolic diseases (Hemochromatosis, Ochronosis, and Wilson's disease), endocrine disorders (hyperparathyroidism and diabetes mellitus) as well as post-operative onset OA (Stockwell, 1991; Buckwalter & Mankin, 1997b). Other factors that influence the onset of OA are outlined in Table 1.6.

As previously mentioned, the progression of OA is sometimes described as three overlapping stages that ultimately results in a loss of function (Buckwalter & Mankin, 1997b). In the first stage, there is a disruption of the cartilage framework that results in the increased hydration of the tissue and a decrease in cartilage aggrecan. During the second stage, the chondrocytes respond to the altered state of the matrix with the up-regulation of cartilage synthesis. It is also during this stage (although at the later stages) that chondrocytes proliferate and may manage to 'repair' the cartilage although possibly to an altered state. During the final stage, the chondrocyte response decreases possibly as a result of mechanical cell damage or in response to paracrine/autocrine signalling (Buckwalter & Mankin, 1997b). It has been shown in osteoarthritic tissue there is an up-regulation of proinflammatory cytokines (including IL-1β, IL-4, TNFα; (LeGrand *et al.*, 2001), mediators of inflammation (including Nitric Oxide and prostaglandin E-2; LeGrand *et al.*, 2001) and matrix metalloproteinases (MPPs) that have been shown to influence matrix synthesis or degradation respectively; (Tetlow *et al.*, 2001; Fernandes *et al.*, 2002). The failure of the repair response subsequently leads to the loss of articular cartilage and the progression of OA with the only current solution being surgery.

With reference to this thesis, one of the most important changes that occurs during the onset of OA is the increase in cartilage hydration (Bank *et al.*, 2000). The hydration of cartilage subsequently leads to a decrease in tissue osmolality that therefore results in the swelling of the chondrocytes (Bush & Hall, 2003). It has been shown recently that chondrocytes in OA cartilage have a larger volume (when compared cells in macroscopically 'normal' cartilage) and that there is a positive correlation between volume and the severity of cartilage degradation (Bush & Hall, 2003). Furthermore, when calculating the extent of cell swelling it was found that the chondrocyte volume was greater than that predicted by passive swelling due to cartilage overhydration. A possible explanation to account for the increase in cell volume would be that chondrocytes from OA cartilage do not have the capacity for RVD. Therefore, as cartilage hydration increases as does cell volume and potentially concomitant changes in metabolism that may influence the integrity of the chondrocytes. With this in mind, an understanding of the RVD response using animal models and human tissue (where possible) may lead to the elucidation of this volume increase.

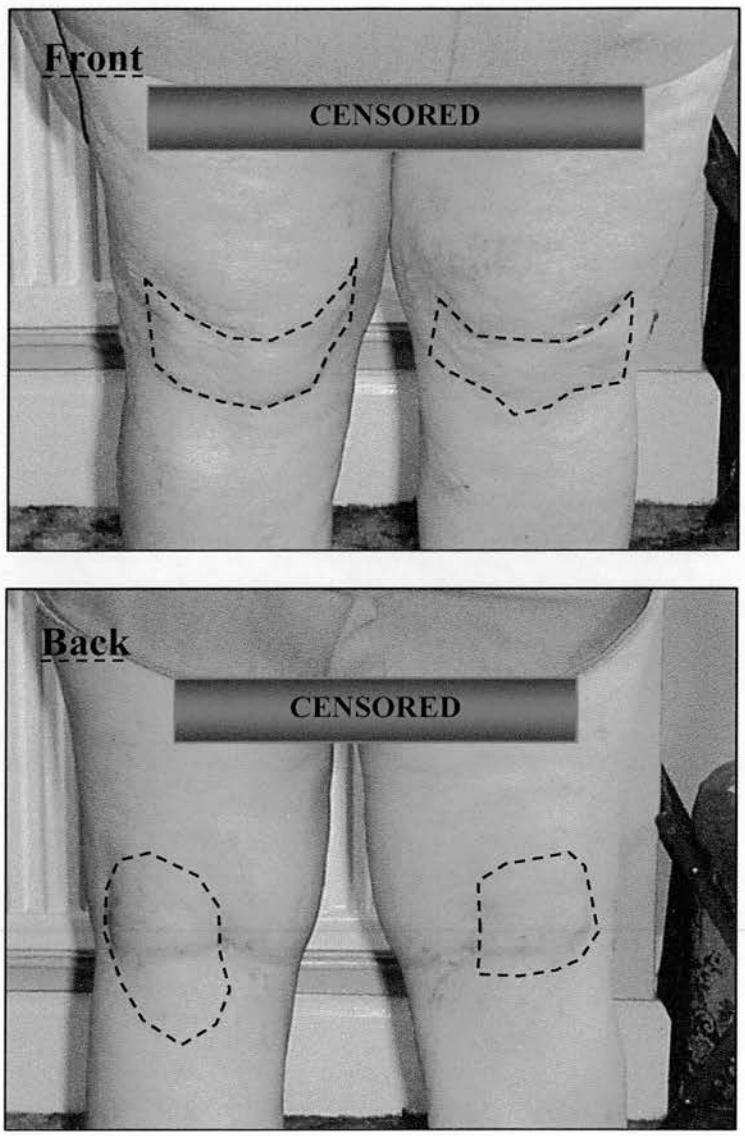


Figure 1.6. External view of a patient with moderate OA.

Photographs were taken using a high quality digital camera of a 78-year-old female patient with moderate OA. From the pictures, it is possible to see areas of swelling (as indicated by the dotted lines) that are indicative of OA.

It

	Influence	Reference
Age	Prevalence Increases with age.	Young *et al.*, 1984; Pai *et al.*, 1997
Weight	Increasing OA with obesity.	Anderson & Felson, 1988; Felson & Chaisson, 1997
Gender	Females more likely to get knee OA where as in Hip OA there is no gender correlation.	Kellgren & Moore, 1952; Felson, 1998
Genetics	RFLP analysis on COL2A1 gene. Mutation in promoter.	Martin & Buckwalter, 2003; Xu *et al.*, 2003;Yao *et al.*, 2003; Eyre, 2002; Han *et al.*, 2002; Helminen *et al.*, 2002; Vikkula & Olsen, 1996
Exercise	Physical exercise i.e. skiing, rugby, skating increases the likelihood of OA.	Kujola *et al.*, 1995; Spector *et al.*, 1996;
Trauma	Acute trauma increases the likelihood of OA.	Roos *et al.*, 1998; Roos *et al.*, 1995
Surgery	Joint surgery increases the chance of OA in that joint in the future.	Roos *et al.*, 1998
Profession	More physical work increases the likelihood of OA for example farm and building work.	Vingard *et al.*, 1991; Croft *et al.*, 1992

Table 1.5. Possible causes of Osteoarthritis.

Osteoarthrosis is classified as a syndrome with many symptoms and risks associated with the onset of the disease. The above Table outlines of the factors that may be involved in the onset or progression of OA.

1.11.0 Aims of this thesis

This thesis aims to examine the possible role of intracellular calcium ($[Ca^{2+}]_i$) as a mediator of both Regulatory Volume Decrease (RVD; Chapter 3) and Regulatory Volume Increase (RVI; Chapter 4) in freshly-isolated and 2D cultured chondrocytes. As the organisation of the F-actin cytoskeleton has been shown to change in response to changes in osmolality, the influence of an intact F-actin cytoskeleton upon the RVD response, and the simultaneous hypo-osmotic induced changes in $[Ca^{2+}]_i$ will then be studied (Chapter 5). Finally, as a possible explanation for the increase in chondrocyte volume in fibrillated cartilage, the ability of chondrocytes isolated from osteoarthritic human articular cartilage to volume regulate will be studied and compared to that of chondrocytes isolated from 'non-degenerate' cartilage (Chapter 6).

Methods

2.1.0 Materials.

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Poole, UK) and were of the highest grade possible. The chemicals used are listed below (Table 2.1 to Table 2.4) and are sub-divided into categories of use.

2.1.1 Tissue Culture.

Common name	Catalogue number	Supplying company	Made in	Working concentration
Ascorbic Acid	A-5960	Sigma-Aldrich	DMEM	50μg.ml^{-1}
Foetal Calf Serum (FCS)	16170-078	Gibco, Paisley, U.K.	DMEM	5% (V/V)
Collagenase; Type I	C-0130	Sigma-Aldrich	DMEM	1.0mg/ml
Dulbecco's Modified Eagle's Medium (DMEM)	D-6171	Sigma-Aldrich	N/A	N/A
HEPES	H-3375	Sigma-Aldrich	Saline	N/A
Powdered Dulbecco's Modified Eagle's Medium (DMEM)	D-5523	Sigma-Aldrich	N/A	N/A
DMSO	D-4540	Sigma-Aldrich	N/A	N/A
Penicillin - Streptomycin	P-0781	Sigma-Aldrich	DMEM	50units.ml^{-1} penicillin and 50μg.ml^{-1} streptomycin
Trypsin - EDTA	25300-054	Gibco, Paisley, U.K.	N/A	N/A
Trypan blue 0.4%	T-8154	Sigma-Aldrich	N/A	N/A
Versene 1:5000	15040-033	Gibco, Paisley, U.K.	N/A	N/A

Table 2.1. Tissue culture reagents.

2.1.2 Fluorophores.

Common name	Catalogue number	Supplying company	Made in	Working concentration
Calcein AM	C-1430	Cambridge Biosciences, Cambridge, U.K.	DMSO	5μM
Fura -2 AM	F- 1225	Molecular Probes, PoortGebouw, The Netherlands	DMSO	5μM

Table 2.2. Fluorophores used.

2.1.3 Pharmacological Agents.

Common name	Catalogue number	Supplying company	Made in	Working concentration
Bumetanide	B 3023	Sigma-Aldrich	Saline	75μM
Dantrolene	251680	Cambridge Biosciences, Cambridge, U.K	DMSO	30μM
EGTA	E 8145	Sigma-Aldrich, Poole, UK	Saline	2mM
Gadolinium	G 7532	Sigma-Aldrich	Saline	100μM
Histamine	H 7250	Sigma-Aldrich	Saline	100μM
Latrunculin B	428020	Sigma-Aldrich	DMSO	10μM
REV 5901	554718	Calbiochem-Novabiochem, Nottingham	DMSO	50μM
Tamoxifen	T 9262	Sigma-Aldrich	DMSO	10μM

Table 2.3. Pharmacological Agents.

2.1.4 Agents Used to Study the Cytoskeleton.

Common name	Catalogue number	Supplying company	Made in	Working concentration
Acetone	A-4206	Sigma-Aldrich	N/A	N/A
Bovine Serum Albumin	A-4378	Sigma-Aldrich	PBS	1%
DNase-Texas Red	D-972	Molecular Probes, PoortGebouw, The Netherlands	PBS	2µl/ml
Paraformaldehyde	P-6148	Sigma-Aldrich	ddH_2O	4%
Phalloidin-FITC	P-5282	Sigma-Aldrich	PBS	25µl/ml
PBS	P-4417	Sigma-Aldrich	ddH_2O	N/A
Tween -20	P-1379	Sigma-Aldrich	N/A	N/A

Table 2.4. Reagents used to study the cytoskeleton.

2.1.0 Culture Media and Experimental Salines.

The tissue culture media used was Dulbecco's Modified Eagle's Medium (DMEM) containing 50units.ml^{-1} penicillin and 50µg.ml^{-1} streptomycin; 280mOsm.kg H_2O^{-1} DMEM; pH 7.4 (95%:5%, air:CO_2). For work on isolated chondrocytes, the osmolality of the media was adjusted to 380mOsm.kg H_2O^{-1} by the addition of 50mM NaCl and the osmolality measured using a vapour pressure osmometer (Wescor Vapro™ 5520). The medium of 380mOsm.kg H_2O^{-1} was used as it has previously been shown that the osmolality of cartilage is between 350-450mOsm.kg H_2O^{-1} (Hall *et al.*, 1996; Wilkins *et al.*, 2000b) and by isolating into a medium within this range would help to limit any

chondrocyte volume changes that may occur during their release from the cartilage matrix.

All fluorescent experiments were performed using a 'basic' physiological saline. This contained (in mM):- HEPES N- [2-Hydroxethyl] piperazine-N'-[2-ethanesulphonic acid) 15; Glucose 10; KCl 5; $MgCl_2$ 1; $CaCl_2$ 1; NaCl 170; pH 7.4; 380mOsm.kg H_2O^{-1}; subsequently referred to as 'control saline'. As with the DMEM, the osmolality of all experimental salines were measured using a vapour pressure osmometer (Wescor Vapro™ 5520).

When either a hypo or hyper-osmotic challenge was applied, the NaCl concentration of the saline was adjusted (by either an increase or a decrease) with all other constituents maintained at the same concentration.

2.2.0 Chondrocyte Isolation and Culture.

Full depth, load bearing cartilage explants were excised aseptically from washed metacarpal and metatarsal phalangeal joints of freshly slaughtered cows (obtained from the local abattoir; used within 4 days) or from human tibial plateaus (obtained with ethical permission; see section 2.6.0) into 280mOsm.kg H_2O^{-1} DMEM. The explants were then incubated overnight (18hours) in 1mg/ml type-I collagenase (37°C; 95%:5% air:CO_2) releasing the chondrocytes into 380mOsm.kg H_2O^{-1} DMEM (37°C; pH 7.4). A DMEM of 380mOsm.kg H_2O^{-1} was used for the cell isolation (as well as for experimentation) as this is closer to the osmolality of cartilage and therefore helps to

decrease any volume changes that may occur if the osmolality of the media were not adjusted (Hall *et al.*, 1996b).

After 18 hours, and to remove any undigested material, the cell suspensions were passed through a sterile (autoclaved) tea strainer and then through a 40μm (sterile) cell-strainer. The suspensions were then centrifuge-washed twice in 380mOsm.kg H_2O^{-1} DMEM at 210g for 10mins (18°C) to remove any remaining collagenase and clumps of cells. The cells were subsequently re-suspended into 380mOsm.kg H_2O^{-1} DMEM, aliquoted onto autoclaved 22mm glass coverslips (contained within stainless steel rings) and used within 2-8 hours.

Chondrocytes to be maintained in longer-term culture were isolated as described above. After the second wash, the cells were re-suspended in 'supplemented media' (DMEM supplemented with 50units.ml^{-1} penicillin and 50μg.ml^{-1} streptomycin; 10% FCS; 50μg/ml Ascorbic acid; 380mOsm.kg H_2O^{-1}) and gently triturated using a pipette. The suspension was then plated at a density of $5x10^6$ cells/ml onto a 25cm^3 flask and incubated at 37°C 95%:5% air:CO_2 for 14-21 days. The media was changed when required. For experimentation, the cells were lifted from the plastic 24 hours before use and before any flasked reached more than 80% confluency. Briefly, the supplemented media was removed and the cells washed gently with 3ml of Versene (Ca^{2+} -free solution; 380 mOsm/kg H_2O^{-1} with NaCl; Gibco). The Versene was then removed, 1ml of Trypsin (380mOsm.kg H_2O^{-1}) added and the flask incubated at 37°C; 95:5% air:CO_2 for 2-5mins. This caused cell rounding and their release from the plastic. The suspension was then diluted with DMEM (1:45; 380mOsm.kg H_2O^{-1}), washed by

centrifugation (8mins; 600g), and re-suspended in 'supplemented DMEM'. The suspension was then aliquoted onto 22mm diameter autoclaved glass coverslips (contained within stainless steel rings) under sterile conditions and used as required (usually within 18-48hrs). These cells are subsequently referred to as '2D cultured chondrocytes' although it is important to note that this refers cells that have flattened during culture and not the culture passage number.

2.3.0 Fluorescent Microscopy Experimental Procedure.

2.3.1 System Set-up.

All fluorescent imaging experiments were performed using a Photon Technology International (PTI) ***ImageMaster™*** ratiometric fluorescent imaging system. The system used is detailed in Figure 2.1. Chondrocytes were passaged onto 22mm sterile glass coverslips (contained within steel rings) and then placed onto the temperature-controlled plate (37°C) of an inverted Nikon microscope attached to a PTI ***ImageMaster™*** system. The cells were visualised using an oil-immersion x40 objective and focused to give a clear image close to the mid-plane of the cells. To illuminate the fluorophore, light from a mercury lamp was passed through a monochromator to set the wavelength. The excitation light then went though a dichroic mirror of a particular wavelength depending upon the fluorophore used (Table 2.5) and this was essential to the separation of the excitation and emission light. For example, using fura-2 light <400nm was used to excite the fluorophore with a recorded emission >400nm. Therefore, the 400nm dichroic mirror allowed light less that 400nm to pass and greater than 400nm reflected into the

camera (Table 2.5). When fura-2 loaded chondrocytes were imaged, a 400nm dichroic mirror was used, and when loaded with calcein this was changed to a 500nm mirror.

Fluorescence data was acquired using an 8-bit video camera (at 0.4 Hz) linked to Dell PC computer (200Hz; 128Mb memory) and data acquired using version 1.4 of the system software. To improve further the signal-to-noise ration and thus image quality, x4 averaging was employed, where four concurrent fluorescent images were acquired and then the average used to provide the final image.

The chondrocytes were perfused continuously (~2ml.min^{-1}) and maintained throughout at 37°C through an experiment using a temperature controlled plate. The perfusion system used was a temperature-regulated, gravity fed system that maintained the experimental salines at 37°C. To ensure that the perfusion rate maintained constant, the perfusion apparatus was filled to the same level for each experiment. The apparatus was frequently cleaned, and the tubing replaced to remove any crystallisation (from the experimental salines) that would otherwise influence the fluid flow. The 'in' and 'out' arms of the apparatus were also kept in a constant position to ensure the same depth of fluid within the chamber and thus improve experimental accuracy.

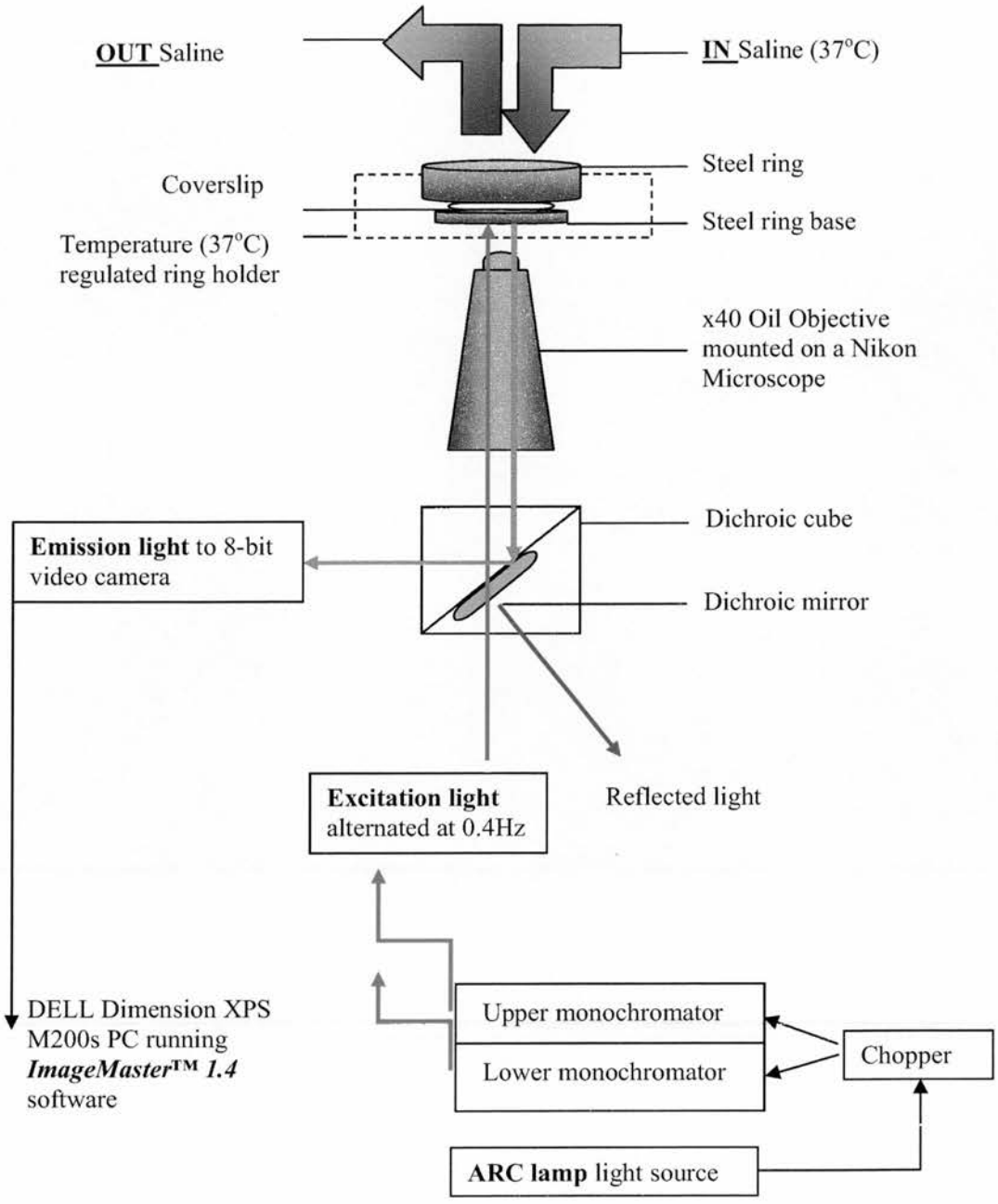


Figure 2.1. System diagram.

All fluorescent imaging experiments were performed using a PTI ***ImageMaster™*** *system. The fluorophore-loaded chondrocytes were passaged onto glass coverslips contained with steel rings and placed into a heated plate on an inverted microscope. The fluorophore was excited by light from lamp (red line) that had passed though a monochromator to set the light to the correct wavelength. Excitation light was also passed thought a dichroic mirror that filtered out light of an unwanted wavelength (blue line) and then the emission light (green line) was collected using an 8-bit video camera. The perfusion apparatus used was gravity fed and temperature regulated to 37°C. Data were collected using a Dell PC running version 1.4 of the* ***ImageMaster™*** *software.*

2.3.2 The Use of Fluorescent Microscopy to Measure Changes in Cell Volume and $[Ca^{2+}]_i$.

Fura-2 (*5-Oxazolecarboxylic acid, 2-(6-(bis(2-(acetyloxy)methoxy)-2- oxoethyl)amino)-5-(2-(2-(bis(2- (acetyloxy)methoxy)-2-oxoethyl)amino)- 5-methylphenoxy)ethoxy)-2-benzofuranyl)- , (acetyloxy)methyl ester)* was developed by Tsien and co-workers in 1985 (Grynkiewicz *et al.*, 1985) and has become one of the most important tools in the study of intracellular calcium signalling (Figure 2.2). Upon the binding of calcium, there is an absorbance wave shift (as measured by the 510nm emission; EM_λ) from the excitation wavelength (EX_λ) of 380nm (an indicator of 'free' calcium) to excitation wavelength (EX_λ) of 340nm (a measure of 'bound' calcium). A ratio of 340:380nm therefore gives a reliable indication of the changes in $[Ca^{2+}]_i$ irrespective of changes in cell volume, intracellular dye concentration or bleaching.

As well as these two calcium sensitive wavelengths, fura-2 can also be excited at ~360nm, termed the isobestic point (Figure 2.2). It is at this point that the fluorophore is insensitive to calcium and can therefore be used to measure changes in cell volume. Using two cultured neural cell lines (N1E-115 & BG105-15), it has been shown that changes in cell volume and $[Ca^{2+}]_i$ using fura-2 can be measured simultaneously (Crowe *et al.*, 1995; Altamirano *et al.*, 1998).

Calcein (*Glycine, N,N'-[[3',6'-bis(acetyloxy)-3- oxospiro[isobenzofuran-1(3H),9'-[9H]xanthene]-4',5'-diyl]bis(methylene)]bis[N-[2-[(acetyloxy)methyoxy]-2-xoethyl]-, bis[(acetyloxy)methyl] ester*) has been shown to be an effective fluorophore for the

measurement of cell volume (Crowe *et al.*, 1995; Bush & Hall, 2000, 2001a, 2001b). Unlike fura-2, it is not excited by UV light and instead has an excitation wavelength (EX_λ) of 495nm with a recordable emission of (EM_λ) 535nm (Figure 2.3). Also unlike fura-2 it has a much greater emission intensity (personal observation), and therefore as well as being suitable for the study of *in situ* chondrocytes is excellent in the study of de-differentiated (and subsequently very thin) chondrocytes.

The theory behind the fluorescent measurement of single-cell volume measurement is dependent upon the fact that once introduced into the cell, the fluorophore is impermeant (Alvarez-Leefmans *et al.*, 1995; Crowe *et al.*, 1995; Altamirano *et al.*, 1998). When a cell loaded with a fluorescent dye is visualised thought a single, focused optical plane, the corresponding recorded emission will remain constant unless the cell is either perturbed or the fluorophore breaks down. Upon the addition of a hypo-osmotic saline, a cell would swell and consequently the amount total amount of fluorophore within the focused optical plane will decrease. This effectively dilutes the amount of the fluorophore within the focused plane and hence a decrease in fluorescent intensity is observed. Should the cell then perform RVD there will be a recovery in fluorescence (Figure 2.4). The reverse is true for cell shrinkage.

Examination of the structures for both fura-2 and calcein it is possible to see that they are charged molecules (hydrophilic) and therefore will not be able to cross the cell plasma membrane easily. Several techniques have been employed to overcome this although the most successful by far is the modification of the fluorophores carboxylic acid groups with AM esters. These esters, when bound to the fluorophore result in the

formation of an uncharged molecule (as well as temporary preventing the fluorescent properties or the molecule) and therefore 'carry' the fluorophore across the plasma membrane. Once inside, the AM ester is cleaved from the fluorophore thus yielding the fluorescent and now cell-impermeant form of the molecule (Tsien *et* al., 1981).

Fluorescence microscopy is not without its problems and some of these are addressed in appendix 1. To summarise, fluorescent microscopy is a powerful tool in the study of cell biology and the elucidation of cell function. Throughout this thesis, the experimental protocols remained constant and so any experimental artefacts or fluorescent nuances would be present in all data and therefore still allow for comparison.

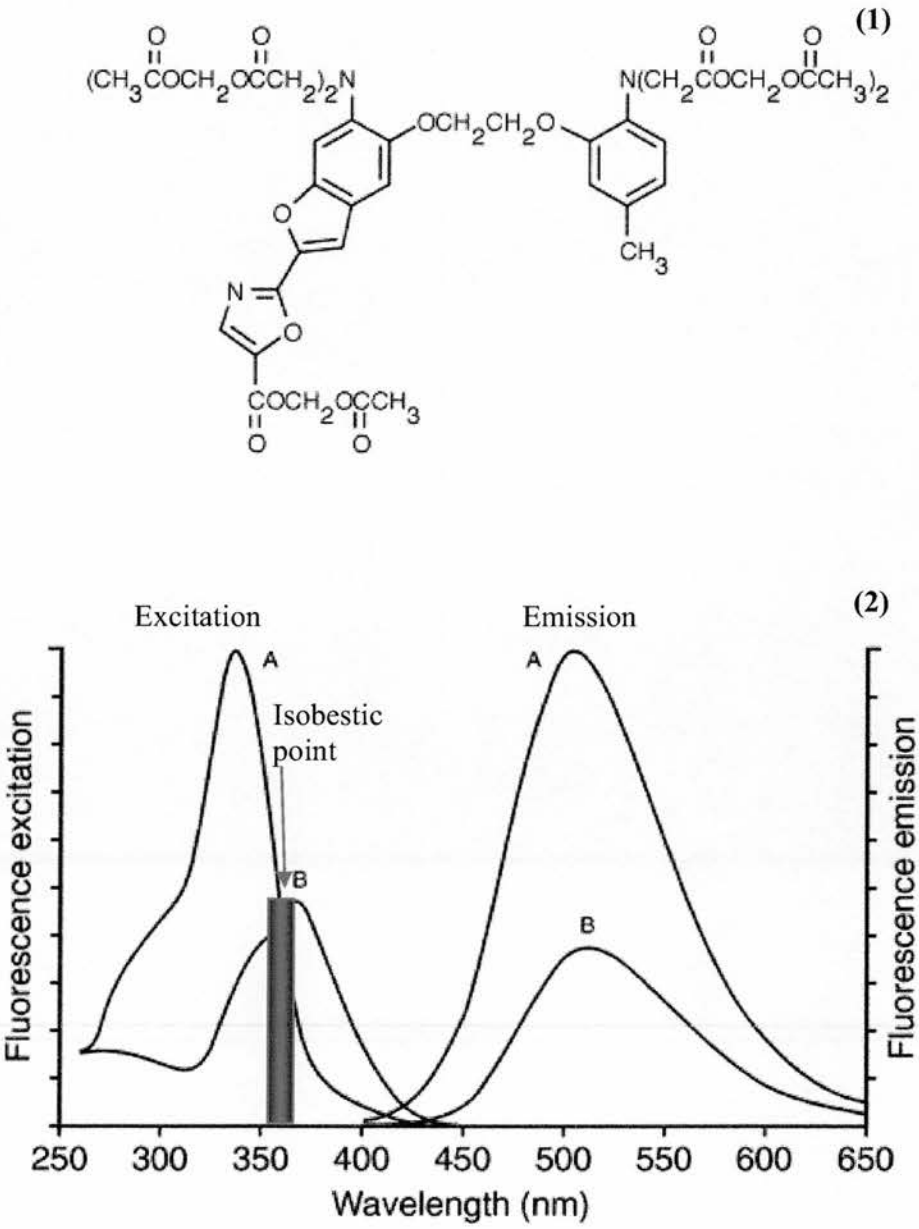
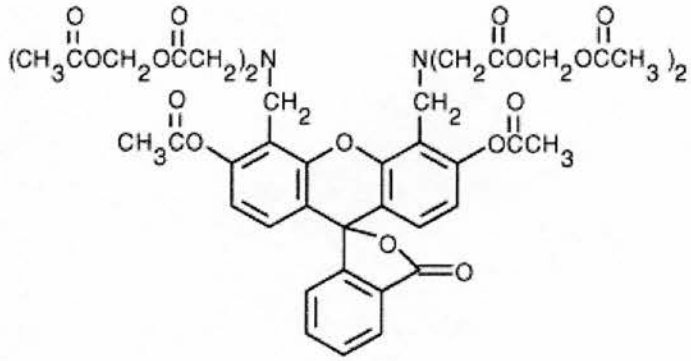


Figure 2.2. Details of Fura-2.

(1) Structure of the cell permeant form, fura-2 AM. (2) Excitation and emission spectra for fura-2. Curves labelled (A) are that of saturated fura-2 with a high 340nm excitation and emission. Curves labelled (B) are that of unsaturated fura-2 with a low 340nm excitation and emission and a slightly higher 380nm excitation and emission. The isobestic point when fura-2 is insensitive to calcium is shown by the red arrow and shaded region.

(1)



(2)

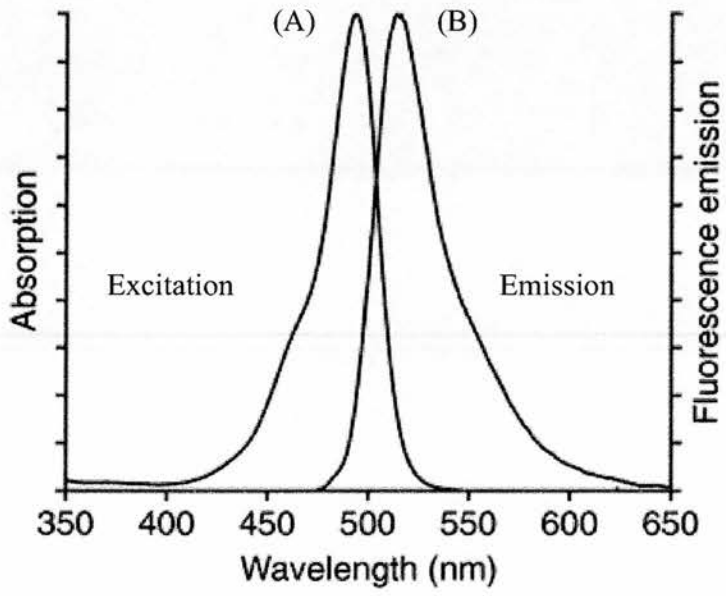
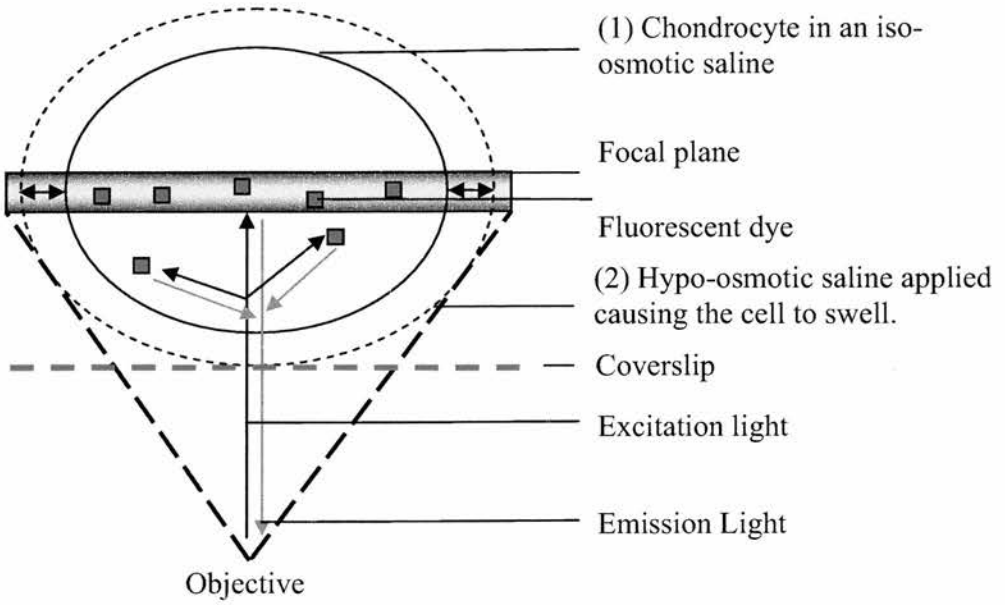


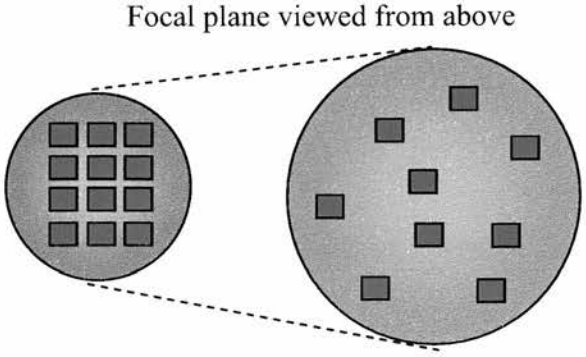
Figure 2.3. Details of Calcein.

(1) Structure of the cell permeant form calcein AM. (2) Excitation and emission spectra for calcein. (A) Excitation curve and (B) emission curve.

(A)


(1) Chondrocyte in an iso-osmotic saline
Focal plane
Fluorescent dye
(2) Hypo-osmotic saline applied causing the cell to swell.
Coverslip
Excitation light
Emission Light
Objective

(B)


Focal plane viewed from above

(C)

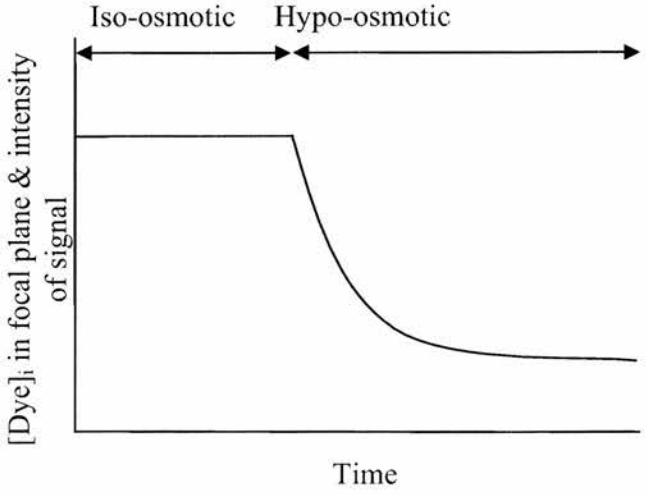

Iso-osmotic
Hypo-osmotic
[Dye]i in focal plane & intensity of signal
Time

Figure 2.4. Theory of fluorescent volume measurement.

(A) Diagrammatic representation of a single chondrocyte. (1) Cell under iso-osmotic conditions. The cell is focused close to the mid-plane and the fluorophore excited by light of a certain wavelength through the objective lens. The fluorescent microscope system does not achieve true confocality (where the excitation light only stimulates the fluorophore in a particular defined region) and therefore as well as the fluorophore within the optical plane emitting 'out of focus' fluorophore will also be stimulated although to a lesser degree. This will also detected by the camera. (B) Upon a hypo-osmotic challenge the cell increases in volume and the fluorophore within the optical planes is effectively 'diluted'. The 'dilution of the fluorophore results in a decrease in signal intensity and is therefore an indicator of an increase in cell volume (C). Should the cell perform RVD the fluorescence would be recovered to that in the iso-osmotic saline.

2.3.3 Dye Loading and Measurement for Fluorescent Microscopy.

Prior to an experiment, and to enable the cells to be visualised by fluorescent microscopy, the cells were incubated with either the fluorescent calcium dye fura-2 AM or the cytoplasmic dye calcein AM (5μM; 30mins; 37°C; pH 7.4). After the incubation period, the coverslips (with chondrocytes attached) were gently washed twice with fresh DMEM (380mOsm.kg H_2O^{-1}) to remove any un-incorporated dye and non-attached/loosely attached cells. The coverslips (contained within steel rings) were placed in a temperature-controlled plate (37°C) and positioned on the stage of an inverted Nikon microscope.

For experiments requiring the measurement of volume in 2D cultured chondrocytes, cells were incubated with calcein AM (5μM; 30mins; 37°C) instead of using fura-2 AM. Calcein was used as preliminary data revealed that probably due to the flat nature of cultured cells, it was not possible to detect volume changes by fura-2. Calcein has a greater fluorescence that fura-2 by two or three times (Kerrigan & Hall unpublished observations) and it was found that is was more suitable for measurements of volume in 2D cultured cells. (Yellowley *et al.*, 2002), have also commented on this, where cell volume measurements were performed on freshly passaged cells to overcome any problems of non-uniform swelling.

2.3.4 Fluorescent Microscopy Procedure.

2.3.4.1 Fura-2.

A fluorescent image was obtained by exciting fura-2 alternately at an excitation wavelength of (EX_λ) 358nm (isobestic point $[Ca^{2+}]_i$ insensitive; volume-sensitive wavelength) and 380nm ($[Ca^{2+}]_i$ sensitive; volume-sensitive wavelength) at 0.4Hz through a 400nm dichroic mirror, with the emission wavelength recorded at 510nm. Regions of Interest (ROIs) were defined around cells and the photometric data recorded within. The use of ROIs decreased the amount of fluorescent data acquired and thus decreased the acquisition time. All images were acquired using an 8-bit video camera linked to Dell PC computer (200Hz; 128Mb memory); for more details see section 2.3.1.

For all experiments an initial 358nm baseline and 358:380nm ratios were obtained in the 'control saline' for 2-3mins and then the experimental saline applied by perfusion. The whole cell response was subsequently recorded (up to 10mins) and the data transferred to Excel™ and MAX™ for analysis as previously described (see section 2.7.0). Using the baseline values recorded in control saline during the first two minutes subsequently allowed for the changes in volume and $[Ca^{2+}]_i$, in response to the experimental condition to be calculated (see section 2.7.0).

2.3.4.2 Calcein.

When using calcein, the dye was excited at 495nm and the emission recorded at 535nm though a 510nm dichroic mirror. The experimental procedure was then as previously described (see above).

2.3.5 Calibration of Fluorescent Microscopy Images.

2.3.5.1 Cell Diameter.

To get an indication of cell diameter, the images acquired by fluorescent microscopy were calibrated using Fluoresbrite™ 10±1.45 μm diameter fluorescent latex beads (Polyscience Inc., Warrington, US). The beads (diluted in control saline) were allowed to attach to 22mm glass coverslips (contained within steel rings) and imaged by fluorescent microscopy as described above. The beads were focused through their mid-plane and visualised using an excitation wavelength of 488nm and an emission wavelength of 510nm where they appeared spherical. Using a sample of 10 beads, the diameters (in μm) were calculated using the PTI Imagemaster™ software and a scale bar created using Adobe Photoshop™.

2.3.6 Single Wavelength Intracellular Fluorescence Changes Linearly with Osmolality.

Before using fura-2 to measures changes in cellular fluorescence as a result of a change in extracellular osmolality, it was important to determine that the changes seen were proportional to the osmotic challenge applied. Chondrocytes were isolated from bovine articular cartilage (as previously described) into a medium of 380mOsm.kg H_2O^{-1} and 30mins prior to an experiment incubated with 5μM fura-2 AM (30mins; 37°C). To prevent any volume regulation by RVD, chondrocytes were also incubated with 50μM REV 5901 (an inhibitor of the chondrocyte osmolyte channel; (Hall & Kerr, 2000) and

75μM bumetanide (inhibitor of the NKCC; Isenring & Forbush, 2001; Liedtke & Cole, 2002).

The chondrocytes were perfused continuously (~2ml.min^{-1}) for 3mins with a control saline of 380mOsm.kg H_2O^{-1} and the 358nm fluorescence (510nm emission; volume sensitive; calcium insensitive) intensity recorded. This provided a steady baseline fluorescence value from which changes could be calculated. The saline was then switched to deliver an osmotic challenge, and the maximal change in fluorescence calculated. Data were then standardised and plotted using a Boyle van't-Hoff plot (Fig. 2.5). From the graph, it is clear to see that over the experiment range the change in intracellular fura-2 fluorescence was linear with changes in extracellular osmolality. There was a high correlation with an r^2 value of 0.97 and a small standard deviation indicating that the maximal volume change for each cell was similar.

The same experiment was then repeated using calcein AM-loaded chondrocytes (excited at 495nm and emission recorded at 510nm) and it was found, as with fura-2, there was a linear change in fluorescence correlating to a change in extracellular osmolality; data courtesy of Dr A.C.Hall (Fig. 2.5). As seen previously, there was a high correlation (r^2 = 0.99) and a small standard deviation. It is interesting to note that the fluorescence for calcein was greater when compared to fura-2 (data not shown) and that a lower camera gain was required to image the chondrocytes. When comparing the linear relationship between fura-2 and calcein with the changes in extracellular osmolality, no significant difference was found in the gradients ($p>0.05$). This would seem to imply that both are

suitable for volume measurements and comparisons can be performed between experiments using either fura-2 or calcein.

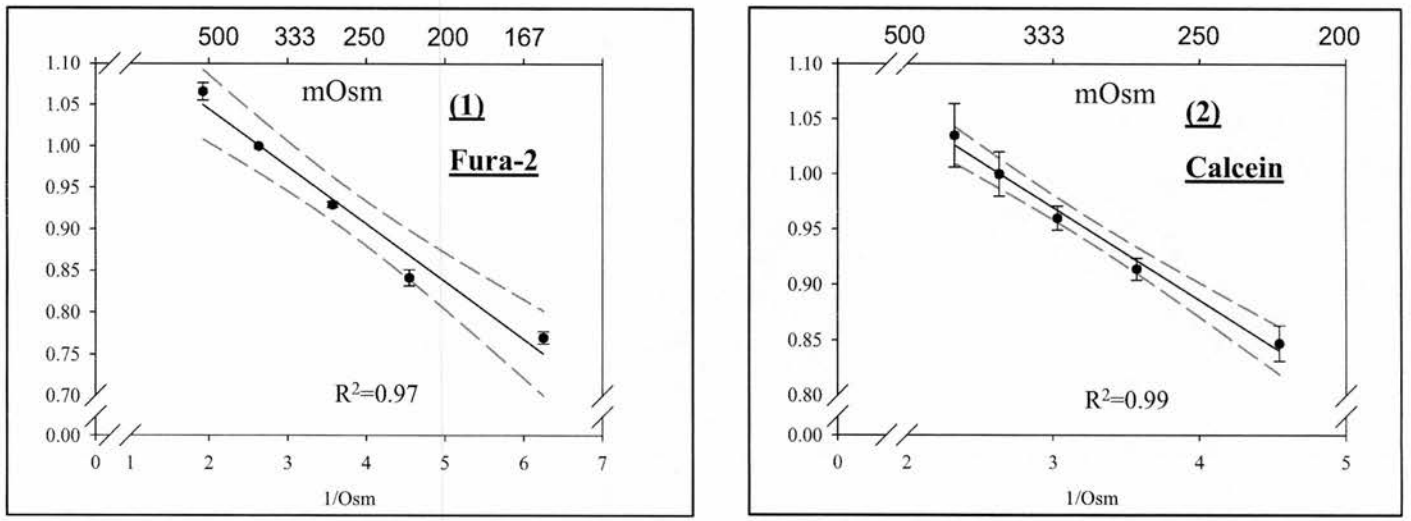


Figure 2.5. The change in intracellular fluorescence correlates with a change in extracellular osmolality.

Chondrocytes were isolated from full depth bovine articular cartilage explants into 380mOsm.kg H_2O^{-1} DMEM and 30mins prior to an experiment incubated with (1) 5μM fura-2 or (2) 5μM calcein. To prevent any volume regulation chondrocytes were also incubated with 50μM REV 5901 (an inhibitor of the chondrocyte osmolyte channel) and 30μM bumetanide (inhibitor of the NKCC). Resting fluorescence was recorded in the control saline for 3mins and the then the saline switched to deliver either a hypo or hyper-osmotic challenge. The maximal change in fluorescence was then calculated and standardised to the control values recorded during the resting period. These values where then plotted against 1/Osm; termed aBoyle Van't-Hoff plot. Over the experimental range, the fluorescence for both fura-2 and calcein changed lineally with that of extracellular osmolality indicating that these fluorophores are suiTable for the study of chondrocyte cell volume. Data are expressed as mean ± s.e.m with 95% confidence intervals shown (blue lines). Calcein data from Dr A.C.Hall, The University of Edinburgh.

2.3.7 Rate of Perfusion.

Having determined that intracellular fluorescence has a linear relationship with the changes in osmolality over the experimental range, it was then necessary to determine the rate of perfusion as different rates may lead to a different cellular response. It has previously been shown that fluid flow initiates a rise in $[Ca^{2+}]_i$ and that the response is dependent upon the rate of flow (Yellowley *et al.*, 1997; Yellowley *et al.*, 1999). It has also been shown that the RVD response can be determined by the rate of the osmotic challenge. For example in trout red blood cells, a slow rate of perfusion (120mins; 15-25%) does not lead to the activation of RVD whereas a faster rate of perfusion initiates the RVD response (Godart *et al.*, 1999).

The perfusion chamber was set up and perfused with 380mOsm.kg H_2O^{-1} 1mM calcium saline as previously described (see section 2.3.4). The saline was the switched to deliver a hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) and 20µl samples removed from the centre of the perfusion chamber every 20 seconds. Data were then plotted and a curve fitted using Sigma Plot 2002 version 8.02 (Fig. 2.6).

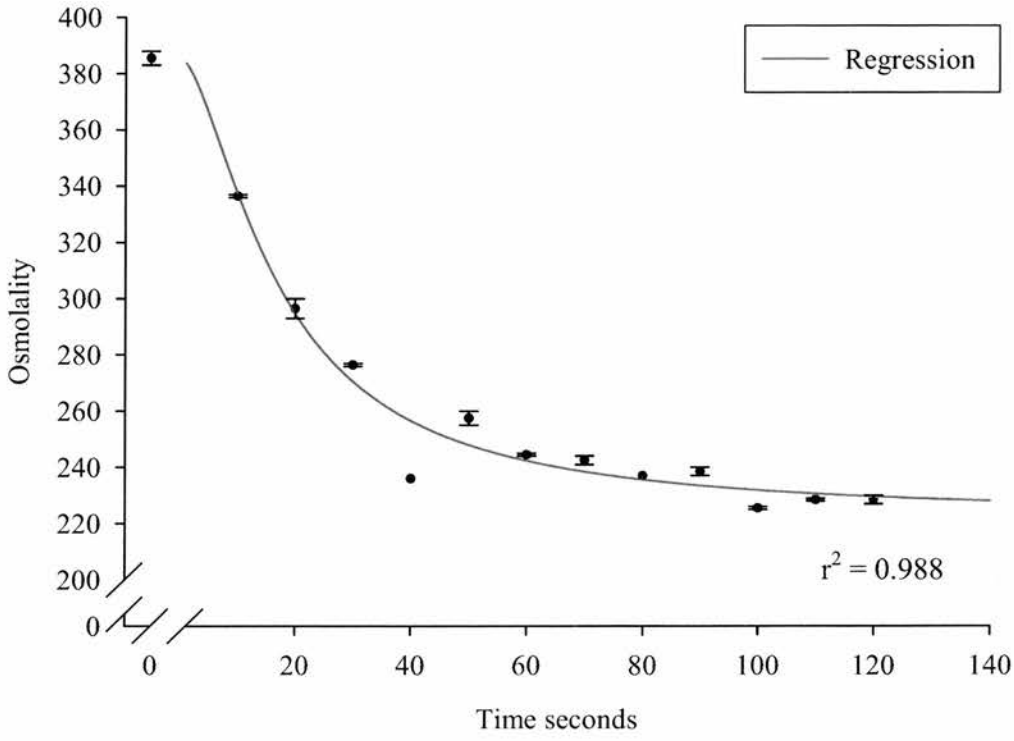


Figure 2.6. Rate of perfusion.

The perfusion chamber was set up and perfused with 380mOsm.kg H_2O^{-1} 1mM calcium saline. The perfusion saline was then switched to deliver a hypo-osmotic saline of 220mOsm.kg H_2O^{-1} and 20µl samples removed from the chamber every 10 seconds. The experiment was then repeated, and data expressed as means ± s.e.m. A non-linear regression curve was then fitted using Sigma Plot with an r^2 value of 0.988. After the perfusion saline was changed, there was a rapid fall in chamber osmolality with almost a complete switch in osmolality after 100 seconds. These data show that the perfusion apparatus is able to deliver a fast and repeatable osmotic challenge.

From the graph, it is possible to see that upon the switch of the perfusion saline, there was a rapid decrease in osmolality and with almost a complete change in under 100 seconds. The small experimental error implies that the method of perfusion was repeatable and thus suitable for the study of chondrocyte volume regulation. To improve experimental accuracy, the position of the perfusion apparatus, and the depth of the perfusion reservoirs (as the system is gravity fed) and the siphon rate were noted and the same values used for all subsequent experiments.

2.3.8 Chondrocyte Autofluorescence.

To ensure that the fluorescence data collected was a result of the fluorophore and not due to cellular autofluorescence, this was tested in non-fura-2 loaded chondrocytes. Chondrocytes were isolated from bovine articular cartilage (as previously described) into a medium of 380mOsm.kg H_2O^{-1} and allowed to settle to 22mm sterile (autoclaved) glass coverslips. The coverslips (contained with steel rings) were placed onto the stage of a Nikon microscope and the cells focused close to the midplane. Cells were then imaged as previously described.

When viewed through the microscope, cells were clearly visible as shown in Figure 2.7a. Conversely, when switched to fluorescent imaging, no cells were visible. Regions of Interest (ROIs) were drawn at random (assuming that due to the high cell density cells would be encompassed) and data recorded. This data is shown in Figure 2.7b and for comparison the 'resting' ratio of fura-2 loaded chondrocytes is also shown. When comparing the auto fluorescence and background data to that of fluorophore-loaded

chondrocytes, the values were significantly less (Student's t-test; $p<0.001$; values are ~0.05%). This therefore this would seem to indicate that cellular autofluorescence is not a major contributor to the fluorescence recorded during experiments and therefore a correction is not needed during analysis.

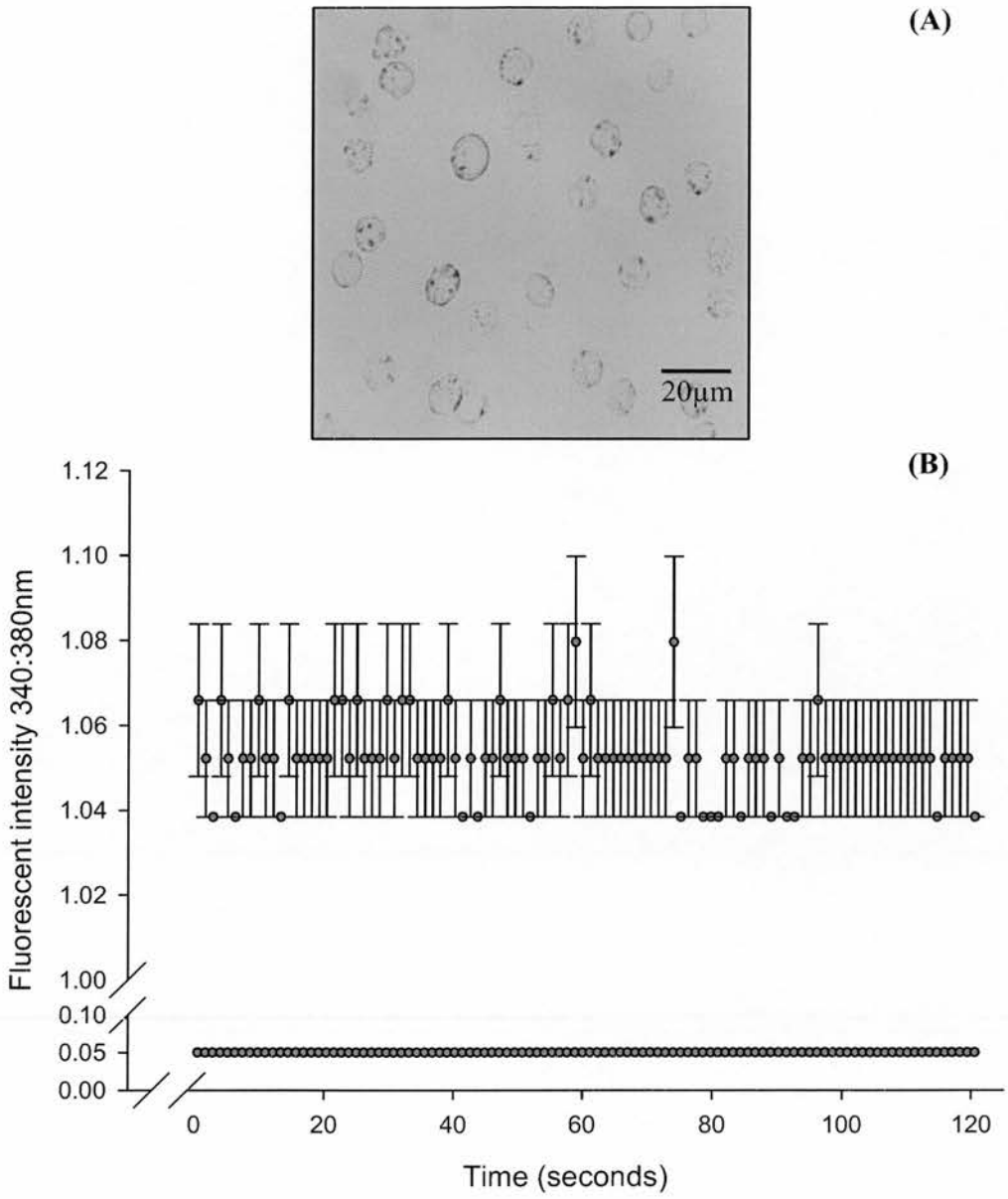


Figure 2.7. Auto fluorescence of isolated chondrocytes.

Chondrocytes were isolated from bovine articular cartilage and a photographed using a Nikon 35mm camera attached to the PTI ***ImageMaster™*** *system (A). (B) Regions of interest were drawn and non-fluorophore loaded chondrocytes were excited at 340nm & 380nm and the data recorded. When compared to the ratio of fura-2 loaded chondrocytes, the ratio of non-loaded chondrocytes was significantly less (~0.05%; $p<0.0001$) and therefore does not seem to contribute to the fluorescent signal of the loaded cells. Date are expressed as mean ± s.e.m. The s.e.m. for the unloaded chondrocytes was zero as the fluorescence was identical for each cell.*

2.4.0 Labelling of the Actin Cytoskeleton.

The actin cytoskeleton has been implicated in RVD in many cells types, yet in chondrocytes, its role is unclear (Cornet *et al.*, 1993; Pedersen *et al.*, 1999; Hoffmann, 2000; Pedersen *et al.*, 2001). To examine the influence of an intact actin cytoskeleton on chondrocyte RVD, cells were incubated with the actin capping agent Latrunculin B (5μM; 37°C; pH 7.4) and the RVD response measured by fluorescent microscopy (see chapter 5). To ensure that the actin cytoskeleton was being disrupted by the Latrunculin, the actin cytoskeleton was fluorescently labelled and visualised by confocal microscopy.

Chondrocytes were passaged onto sterile 22mm (autoclaved) coverslips and were incubated with the drug for 30mins (5μM; 37°C; pH 7.4). Cells were then fixed in ice-cold, 4% paraformaldehyde (made in 380mOsm.kg H_2O^{-1} Phosphate Buffered Saline; PBS) at 4°C for 20mins. After the fixation period, the coverslips were washed x3 in ice-cold 380mOsm.kg H_2O^{-1} PBS to remove any remaining paraformaldehyde and subsequently quenched (to prevent any auto fluorescence of the paraformaldehyde) for 10mins in ice-cold 50mM NH_4Cl (made in PBS; 380mOsm.kg H_2O^{-1}). To remove any remaining NH_4Cl, the coverslips were washed x3 in ice-cold 380mOsm.kg H_2O^{-1} PBS.

The actin cytoskeleton was fluorescently labelled with 25μl/ml phalloidin-FITC (binds to polymerised F-actin; green) and 2μl/ml DNase-Texas Red (binds to monomeric G-actin; red; Figure 2.8). As these dyes are membrane impermeant, the chondrocytes were first permealised (extracted) using ice-cold acetone (10mins at -20°C). After the

extraction, the cells were dipped in 100mM glycine (to stop any further reaction; made in PBS; 380mOsm.kg H_2O^{-1}) and washed x3 in ice-cold 380mOsm.kg H_2O^{-1} PBS. The coverslips were then placed into blocking buffer (1% Bovine serum Albumin; 0.05% Tween 20; made in PBS; 380mOsm.kg H_2O^{-1}) and the fluorophore s subsequently added. The chondrocytes were then incubated in the dark for 45 mins at room temperature.

After the incubation period, the coverslips were washed x3 in 380mOsm.kg H_2O^{-1} PBS-Tween (0.05% Tween 20 V/V) for 15 mins, dipped in pure water (ddH_2O) and then mounted onto glass slides using Mowiol (Calbiochem). The slides were stored at 4°C and viewed within 48 hours by Confocal Laser Scanning Microscopy (CLSM).

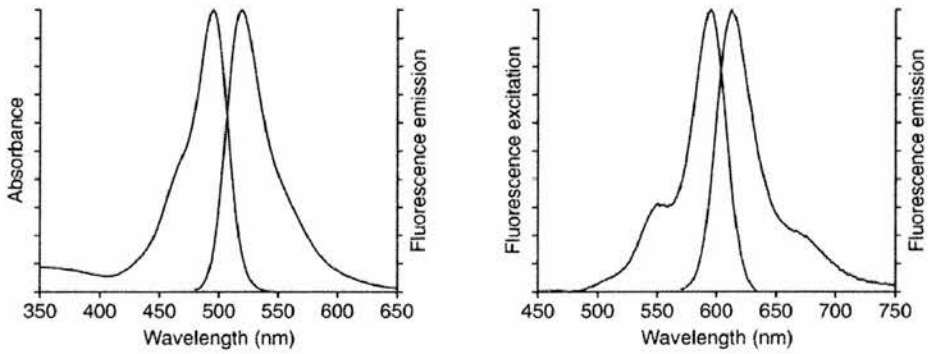


Figure 2.8. Details of the cytoskeletal fluorophores used.

To study the actin cytoskeleton two fluorescent labels were used. To label F-actin, the chondrocytes were incubated with Phalloidin-FITC (green) and the fluorophore excited at 488nm using an Argon laser. To label the G-actin the chondrocytes were incubated with DNase-Texas Red (red) and the fluorophore excited at 520nm using a Helium-Neon laser. As there was a slight overlap in the emission spectra all images were acquired using multi-tracking (see section 2.5.0). Briefly, the process of multi-tracking prevented fluorescent 'bleedthrough' and therefore increased the accuracy of all the acquired images by preventing fluorescent artefact. Figures are from Molecular Probes™.

2.5.0 Confocal Microscopy Experimental Procedure.

Cartilage explants were excised aseptically from full depth bovine articular cartilage explants into 280mOsm.kg H_2O^{-1} DMEM and chondrocytes isolated as previously described (see section 2.2.0). Cells where then either visualised on the day of isolation or cultured for 3 weeks. Prior to visualisation, cells were fixed and the actin cytoskeleton labelled (see section 2.4.0). The cells were then transferred to the stage of a Zeiss Axioskop LSM510 upright confocal microscope system and visualised by Confocal Laser Scanning Microscopy (CLSM).

The Phalloidin-FITC was excited using an Argon laser with an excitation wavelength of 488nm and a bandpass of 500-530nm. The DNase-Texas Red was excited using a Helium-Neon laser with an excitation wavelength of 520nm and a recorded bandpass of 535-590nm (see Fig. 2.8). All images were acquired at 0.6Hz and the power output of each laser adjusted to ensure sufficient fluorophore excitation with no saturation of the emission signal.

When examining the emission and excitation spectra of the fluorophores used in this study, there was the potential for bleedthrough between the two used dyes (Fig 2.8). Bleedthrough occurs when there is an overlap in the emission of two fluorophores as the emission of one fluorophore (excited at the lower wavelength) is detected in the bandpass of the second. This can therefore lead to false images and the incorrect interpretation of an experimental situation. This is a particularly important consideration when performing co-localisation experiments or when examining images comprised of

more than one fluorophore. To avoid this, an acquisition process termed 'Multi-tracking' was employed.

The process of 'Multi-tracking' prevented signal bleedthrough whereby each fluorophore was excited concurrently (as opposed to simultaneously) and dichroic filters were automatically placed in front of the detection path. This prevented signal bleedthrough from one channel into the next and thus provided data free from fluorescent artefact. Using this method, one can be assured that the image acquired at a particular wavelength was due to the fluorophore emitting within the defined bandpass and not due to bleedthrough from a second fluorophore.

All images were acquired at a resolution of 1024x1024 pixels using an optimal pinhole size calculated to match the size of the Airy disk. Briefly, an Airy disk was defined mathematically by Sir George Airy (19^{th} century Astronomer) and describes the concentric, progressively fainter circles of light radiating from a central point of maximum intensity formed by the reinforcement and diffraction of light when passed through an aperture. This therefore limits the angular resolution of a lens where an object larger than the Airy disk will be blurred. By adjusting the pinhole size, the maximum amount of data was acquired without trying to sample data that is physically impossible due to the optical setup (termed 'over sampling'). This adjustment allowed for the acquisition of the optimal amount of 'in focus' light and therefore decreased experimental inaccuracy (Dr Marek Kukula; The University of Edinburgh; personal communication). There was a slight mis-match in the pinhole calibration as FITC had a smaller optimal size when compared to DNA-Texas Red. As it was advised to keep the

pinhole (and subsequently the optical sections) the same for each fluorophore, the pinhole size for the FITC was slightly increased to match the DNA-Texas Red.

For the acquisition of high-powered zoomed images, data were acquired using a higher imaging frequency so that it was twice that of the optimum required to capture all periodic components to compensate for the poor axial resolution (Shotton & Sheppard 1997). Images were subsequently de-convolved (a process that corrects for the elongation in the z-plane during image acquisition) during analysis to improve definition and reduce the artificial elongation in the z-axis.

2.5.0.1 Tile Scanning.

When the acquisition of images over a large surface area was required, a technique referred to as 'tile scanning' was used. Chondrocytes were prepared as previously described (see above) and the coverslip placed onto the motorised stage of a Zeiss Akiovert LSM510 inverted confocal laser scanning microscope. The Phalloidin-FITC ($25\mu l.ml^{-1}$) and DNase-Texas Red ($2\mu l.ml^{-1}$) were excited as previously described through an x10 air objective and images acquired though a single optical plane using x4 averaging. After a field of view was imaged, the motorised stage moved the coverslip to an area adjacent to the one just imaged, eventually covering a total area of 4 x 4mm (see Fig 2.9).

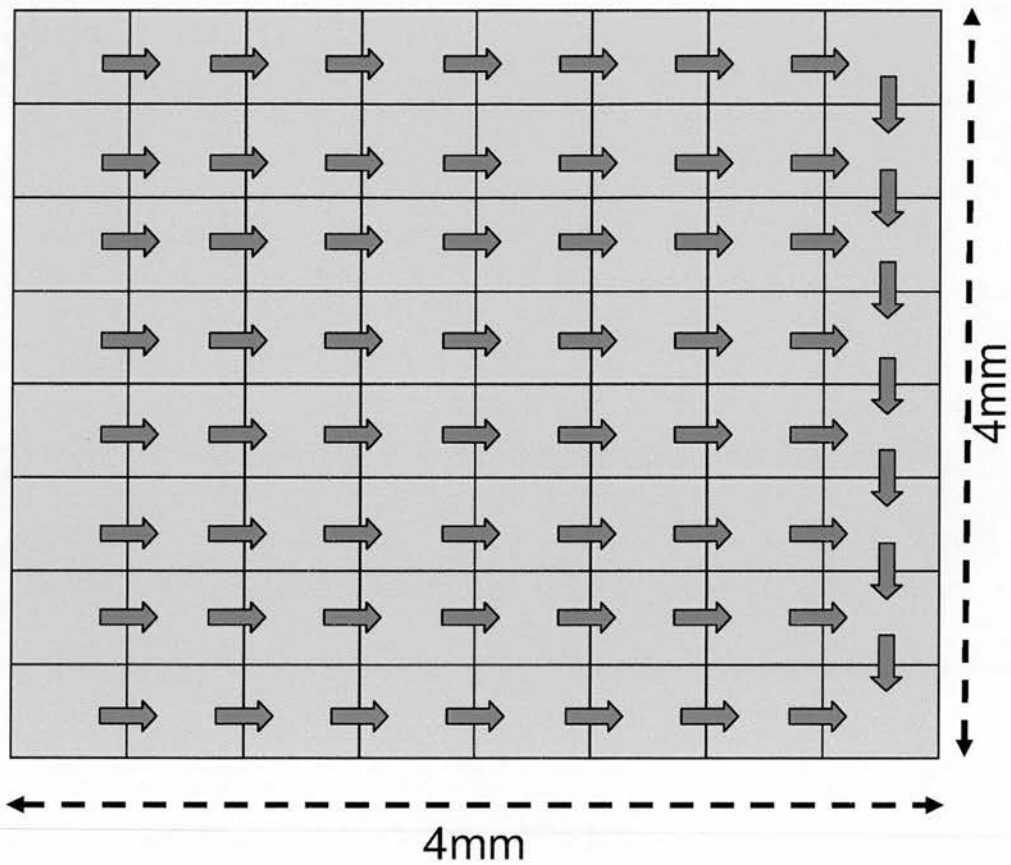


Figure 2.9. Data acquisition during tile scanning.

To image a large area of a coverslip a process referred to as tile scanning was used. Images were acquired using a Zeiss Axiovert LSM510 confocal laser scanning microscope equipped with a motorised stage. Images are acquired through an x10 air objective using x4 averaging at a single z-section. The stage was programmed to move after a field of view has been scanned until the whole defined area (in this case 4mm squared is imaged.

2.6.0 Preparation of Human Tissue.

2.6.1 Tissue Preparation.

To examine the RVD response in human articular chondrocytes, cartilage was obtained (with ethical permission) from the tibial plateau of patients having undergone Total Knee Arthroplasty (TKA) surgery. The patients within this study were in the age range of 47-78 years (average 67 years) with an approximate equal number of joints from male and female patients. Overall, for this chapter 18 knees were used. Unless otherwise stated, patients were healthy (excluding osteoarthritis). The tibial plateau were removed during surgery into sterile 280mOsm.kg H_2O^{-1} DMEM and maintained at 4°C until required (usually within 18 hours).

2.6.2 Cartilage Grading.

In order to compare the RVD response from chondrocytes isolated from areas of 'non-degenerate' and 'degenerate' cartilage, the level of cartilage deterioration was visually assessed and graded on a scale of 0 to 3 according to an increase in degeneration (Collins & McElligott, 1960; Bush *et al.*, 2000). For all experiments, cartilage explants were only removed from the articular surface that resided outside of the joint meniscus (determined by reassembling the joint prior to cutting the explants). Only cartilage from the tibial plateaux were used.

Macroscopically, cartilage from grade 0 appeared 'normal', 'pearly white' in colour with no indications of fibrillation or splitting. Cartilage from grade 1 had a rough

articular surface (when traced using the back of a scalpel blade) and appeared slightly thinner when compared to grade zero cartilage. Cartilage from grade 2 had a rougher surface when compared to grade zero and was noticeably thinner indicating there had been significant erosion of the superficial zone. There was also a loss of the pearly white colour, and the cartilage appeared more 'creamy'. Grade 3 cartilage was increasingly thinner with a loss of the superficial zone and part of the mid-zone. The surface appeared rough and slightly pitted when compared to grade zero (Table 2.6).

Graded level	Experimentally used as	Appearance of cartilage
0	'Non-degenerate'	No macroscopic degeneration. Under confocal microscopy all three major articular cartilage zones (SZ, MZ, DZ) were visible
1	'Non-degenerate'	Rough articular surface, some loss or weakly attached SZ
2	'Degenerate'	Complete loss of SZ
3	'Degenerate'	Complete loss of SZ and damaged/loss of some MZ. Macroscopically soft to the touch

Table 2.5. Summary of the cartilage differences according to the experimental grade.

To examine the RVD response in human articular cartilage, cartilage was obtained from the tibial of patients having undergone Total Knee Arthroplasty (TKA) surgery. Prior to the excision of cartilage explants and chondrocyte isolation, the tibial plateau was graded according to the level of degeneration associated with increasing osteoarthritic pathology. To further show the changes in degeneration cartilage explants were incubated with 5μM calcein AM and 1μM propidium iodide (37°C; 95 %:5 % air: CO_2; pH 7.4) and viewed using Confocal Laser Scanning Microscopy (CLSM). In regions of 'non-degenerate' cartilage (grade 0), the surface appeared pearly white and smooth to the touch with a scalpel. With increasing degeneration, the cartilage became thinner due to the partial loss of the superficial zone (grade 1); loss of the superficial zone (grade 2); and loss of the superficial zone plus part of the mid zone (grade 3). Cartilage explants excised from grades 0 and 1 cartilage were experimentally used as 'non-degenerate' and those from grades 2 and 3 were used as 'degenerate'. SZ= Superficial Zone, MZ= Mid Zone, DZ = Deep Zone.

2.6.3 Cartilage Grading by Confocal Laser Scanning Microscopy (CLSM).

To distinguish further the differences in the cartilage grades, the explants were visualised by Confocal Laser Scanning Microscopy (CLSM; see section 2.5.0). Cartilage explants were excised aseptically (as previously described) into 280mOsm.kg H_2O^{-1} DMEM and washed twice with fresh media to removal any synovial fluid. Thirty mins prior to an experiment, the explants were placed into 1ml of fresh 280mOsm.kg H_2O^{-1} DMEM and incubated with 5μM calcein AM (indicator of morphology) and 1μM propidium iodide (indicator of dead cells; 37°C; 95 %:5 % air: CO_2; pH 7.4). After the incubation period, the explants were washed with fresh 280mOsm.kg H_2O^{-1} DMEM (to remove any un-incorporated dye) and attached to 60mm plastic dishes by Superglue. The superglue was applied to the subchondral bone as this was far removed from the point of visualisation during microscopy. The explants were then submerged in 280mOsm.kg H_2O^{-1} DMEM and placed onto the stage of a Zeiss Axioskop LSM510 microscope. When visualised and where present, it was possible to observe the three distinct cartilage zones, superficial (SZ), mid (MZ) and deep (DZ) and detect any morphological differences relative to the grade. Cartilage from grade 1 had a rough articular surface and some of the surface zone missing or weakly attached. In grade 2, the cartilage was more degenerate with a complete loss of the articular surface. In grade 3 cartilage, there was a complete loss of the SZ and the MZ was partially missing (Fig 2.10).

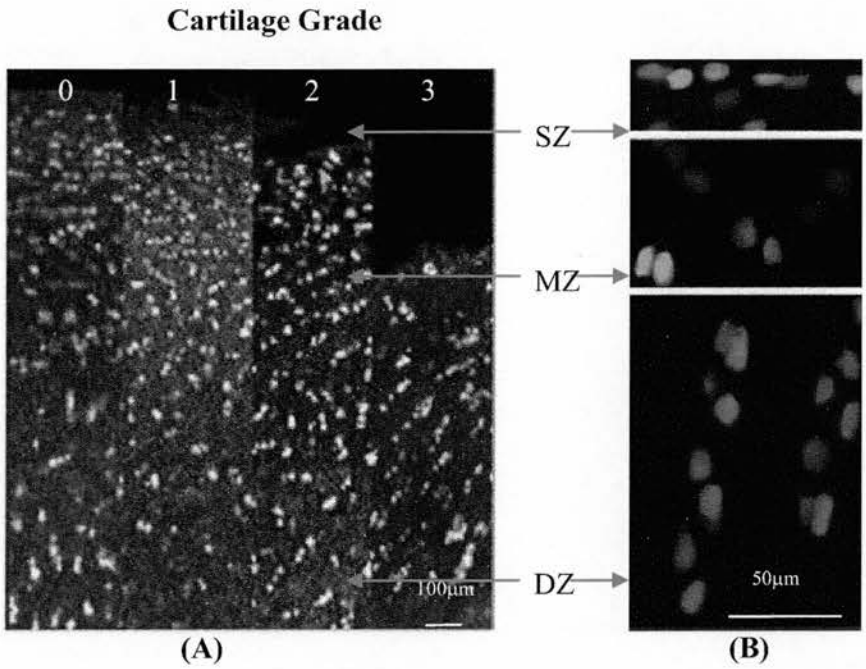


Figure 2.10. Confocal images illustrating the grading of human articular cartilage.

Cartilage explants were excised aseptically (as previously described) into 280mOsm.kg H_2O^{-1} DMEM and washed twice with fresh media to remove any synovial fluid. The explants incubated with 5µM calcein AM and 1µM propidium iodide and viewed using Confocal Laser Scanning Microscopy (CLSM; for methods see section 2.5.0). (A) Transverse sections of human articular cartilage were acquired using an x10 air objective where it was possible to observe the three distinct cartilage zones, superficial (SZ), mid (MZ) and deep (DZ). With increasing degree of degeneration associated with osteoarthritis, there was a loss of the superficial (SZ) and mid zones (MZ). (B) High powered images of calcein loaded in situ chondrocytes. Using an x63 emersion objective images of in situ chondrocytes were acquired. It was possible to observe the difference in chondrocyte distribution, orientation and size in between the different cartilage zones. In the SZ, chondrocytes were smaller, parallel to the articular surface when compared to cells in the other zones. In the MZ the cells were larger and reside as pairs. In the DZ the cells were larger still and are often found in stacks perpendicular to the articular surface. Image courtesy of Dr P.G.Bush; The University of Edinburgh.

2.7.0 Data Analysis.

2.7.1 Representation.

All data are expressed as a mean and standard error of the mean (s.e.m; calculated between joints and not cells) and statistical analyses performed using SigmaStat and SigmaPlot (Jandel Scientific, Ekrath, Germany) and Excel (Microsoft® 2002). Volume (represented by the EX_λ of 358nm and the EM_λ of 510nm) and $[Ca^{2+}]_i$ (represented by the 358:380nm ratio) were standardised to the basal levels (i.e. fluorescent intensity in an iso-osmotic, non-experimental saline) recording during the 'resting' period. This subsequently allowed for fluorescent changes in response to the experimental condition to be calculated and comparisons between experimental groups to be performed. For all experiments the volume and $[Ca^{2+}]_i$ data were standardised for each individual cell using each cell's individual 'resting' baseline. This subsequently improved accuracy and decreased analytical error. The number of joints is shown as 'n' and the number of cells 'N' is also given. For statistical analysis the number of joints was always used as this took into account the natural variation between each joint as well as between each cell.

2.7.2 'Responding' and 'Non-responding' Experimental Groups.

To allow comparisons between chondrocytes that did perform RVD and those that did not, the chondrocyte population was divided into two groups. The first group, subsequently termed 'responding' comprised of chondrocytes showing greater than 50% RVD when calculated as recovery from the maximal change in volume (V_{max}) to the end of the experimental period (V_{final}). The second group, subsequently termed 'non-

responding' comprised of chondrocytes showing less than 50% RVD when calculated as recovery from the maximal change in volume (V_{max}) to the end of the experimental period (V_{final}). For both experimental groups, the experimental period was always 10mins (see Fig. 3.3. for an example of a chondrocyte performing RVD).

2.7.3 Data Analysis of the Actin Cytoskeleton

To quantify the actin cytoskeleton in freshly-isolated chondrocytes (images acquired by CLSM), a method from Guilak *et al.,* (2002) was adapted and employed. It has previously been shown in both human and bovine articular chondrocytes that polymerised (F) actin is located cortically (Langelier *et al.*, 2000; Zwicky & Baici, 2000b) and therefore when examining a linear profile drawn through the centre of a cell, the highest fluorescent intensity will be towards the periphery. It is noted that the polymerised actin was not completely uniform and within the cortical arrangement there are always areas of higher fluorescent intensity (Kerrigan & Hall unpublished observations). The method of analysis needed to take these facts into consideration.

To allow for repeatable and unbiased analysis, two linear profiles through the centre of the cell (parallel in the z-plane and perpendicular in the x-y planes) were calculated (using Zeiss Image Examiner software). These were then averaged to give a 'linear cell profile'. The background fluorescence was then measured (from areas with no FITC/Texas Red fluorescence) and this subtracted from the 'linear cell profile'. To standardise the data (as the cells are of different sizes), all profiles were divided by the average cell diameter (calculated using Zeiss Image Examiner) to give a final

standardised cell fluorescent profile. Statistical analysis was performed using Sigma Stat (Jandel Scientific, Ekrath, Germany) were a p value of less than 0.05 was considered significant. When comparing experimental groups, a Student's unpaired, t-test was performed whereas when comparing cortical to central fluorescent intensity a paired Student's t-test was used. All graphs were plotted as means ± standard error of the mean (s.e.m) where appropriate.

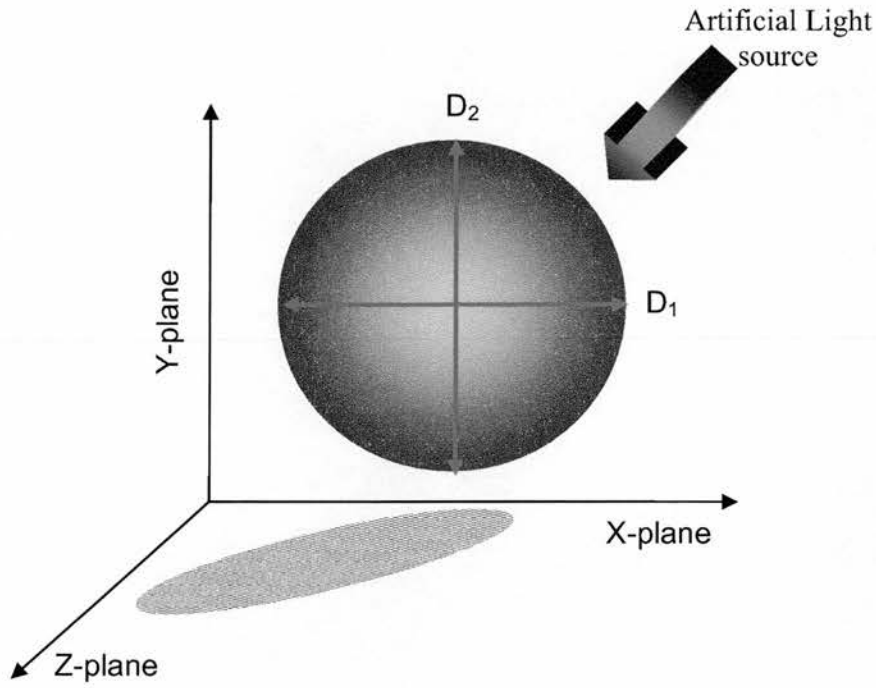


Figure 2.11. Diagrammatic representation of the technique used to quantify the actin cytoskeleton confocal images.

The actin cytoskeleton was labelled with Phalloidin-FITC (F-actin; green) and DNase Texas Red (G-actin; red) as previously described (see Materials and Methods) and cells imaged by confocal microscopy. To quantify the effects of Latrunculin B on the actin cytoskeleton a method of linear profiling (adapted from Guilak et al., 2002) was adapted, modified and employed. Images were acquired by CLSM and the image that was closest through the middle of the cell (calculated as half the distance from the top to the bottom of the cell in the z-plane) was used for the profiling. To standardise the technique (and to prevent bias), two perpendicular profiles were drawn (using the Zeiss imaging software) in the x-y planes (D_1 & D_2; always vertical and horizontal) and then averaged to give a 'cell linear profile'. As cells are different sizes, the 'cell linear profiles' were subsequently standardised to the diameter of the cell. Data were then transferred to Excel (Microsoft© 2002) and Jandel Sigmaplot (Jandel Scientific, Ekrath, Germany) for analysis.

2.7.4 Statistical Tests.

The means of all experiments (where applicable) were compared using an unpaired Student's t-test and a significant difference was accepted when $p<0.05$. Differences in sample variance were compared using an f-test where a significant difference was accepted when $p<0.05$. Population studies were performed using a χ^2 test. All significant differences are represented in all figures as * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) respectively. The number of joints (n) and cells (N) per experimental group are also given. Linear regression analysis was performed by extrapolation of either the 358nm or the 495nm data using Sigma Plot and Sigma Stat (Jandel Scientific, Ekrath, Germany). This was then used to calculate the rate of RVD (expressed as a t½; where a t½ is half the time required for volume to be recovered by 50% from the maximal change over the 10min experimental period). An r^2 >0.90 was nominated as a significant fit.

Regulatory Volume Decrease (RVD)

3.1.0 Chapter Introduction

In response to a decrease in extracellular osmolality, many cells types have the capacity to volume-regulate via a process termed ***Regulatory Volume Decrease*** (RVD; Hoffmann, 1992; Hoffmann & Dunham, 1995; O'Neill, 1999). RVD is the process where the cell 'dumps' osmolytes and consequently looses osmotically obliged water. This subsequently mediates the recovery of cell volume back towards the initial pre-determined set-point. As previously mentioned (section 1.6.0), there are several transporters that are potentially capable of mediating this response including BK and SK K^+ channels, KCC cotransporter, Cl^- channels and a Volume Sensitive Anion Channel; (VSAC). The capacity for RVD has been shown in both freshly-isolated and *in situ* articular chondrocytes although the mechanism is still unknown (Bush & Hall, 2000, 2001b).

A possible mediator of the RVD response is a change in $[Ca^{2+}]_i$ (O'Neill, 1999; Jakab *et al.*, 2002) and in chondrocytes it has been shown that mechanical stimulation (including fluid flow, membrane deformation and changes in osmolality) can initiate a transient rise in $[Ca^{2+}]_i$ (Yellowley *et al.*, 1997; Yellowley *et al.*, 1997; Guilak *et al.*, 1999; Guilak*et al.*, 1999; Yellowley *et al.*, 2002). The role for calcium in chondrocyte RVD and a review of the literature indicated that the role is dependent upon the cell type. For example in rat cerebellar neurons it has been shown that RVD is completely independent of a the associated rise in $[Ca^{2+}]_i$ (Moran *et al.*, 1997) where conversely, in proliferating prostate tumour spheroids RVD was inhibited by the block of the associated rise in $[Ca^{2+}]_i$ (Sauer *et al.*, 1998).

Therefore, in order to further elucidate the signalling pathways involved in the RVD response, single cell fluorescent microscopy was used to simultaneously study changes in $[Ca^{2+}]_i$ and cell volume under various experimental conditions in response to a hypo-osmotic challenge. Furthermore, as previous work has shown that time in 2D culture can influence chondrocyte morphology and products of synthesis (Benya & Shaffer, 1981; Benya *et al.*, 1988), comparisons were performed between freshly-isolated chondrocytes with that of cells maintained in 2D culture for 3 weeks.

Hypothesis to be tested

Both freshly isolated and 2D cultured chondrocytes depend upon a stretch-sensitive rise in $[Ca^{2+}]_i$ to mediate RVD.

3.2.0 Results

3.2.1 Images of Isolated Fura-2-Loaded Chondrocytes.

Chondrocytes were isolated from full-depth bovine articular cartilage explants (as previously described; see Materials and Methods; section 2.2.0) and 30mins prior to an experiment, were incubated with fura-2 AM (5μM; 37°C). The non-fluorescent acetoxymethyl (AM) form of the dyes diffuses rapidly across the plasma membrane and the AM ester-group is cleaved by intracellular esterases resulting in the release of the cell-impermeant fluorescent form (Grynkiewicz *et al.*, 1985). For freshly-isolated chondrocytes, this period also allowed the cells to attach to the glass coverslips.

Cells were then transferred to a heated stage (37°C) of a Nikon microscope and images acquired using a PTI ***Imagemaster™*** system through an x40 oil objective focused through the midplane of the cells. The incorporated fura-2 was excited through a 400nm dichroic mirror at alternating wavelengths of 358nm (Isobestic point $[Ca^{2+}]_i$ insensitive; volume-sensitive wavelength) and 380nm (Volume-sensitive; $[Ca^{2+}]_i$ sensitive; volume sensitive wavelength)at 0.4Hz The emission was recorded at 525nm. All images were acquired using x4 averaging to decrease experimental noise.

When visualised, the freshly-isolated chondrocytes appeared spheroidal with an apparent heterogeneity in cell size (Fig. 3.1). Throughout the main body of the cell, the dye appeared to be evenly distributed, as there no areas of puntate fluorescence were visible. This would imply that there was little or no compartmentalisation that would have otherwise hindered the detection of changes in cell volume and $[Ca^{2+}]_i$. At this

stage, it is important to note the limitations in the fluorescent microscope system. It is possible that the size of the optical plane used, in conjunction with the resolution of the machine may not have been able to detect small amounts of fura-2 sequestering. Although, as it has previously been shown that the fura-2 fluorescence changed linearly with osmolality (see section 2.3.6), it was assumed that there was little sequestering of the fura-2 into the non-osmotically sensitive space.

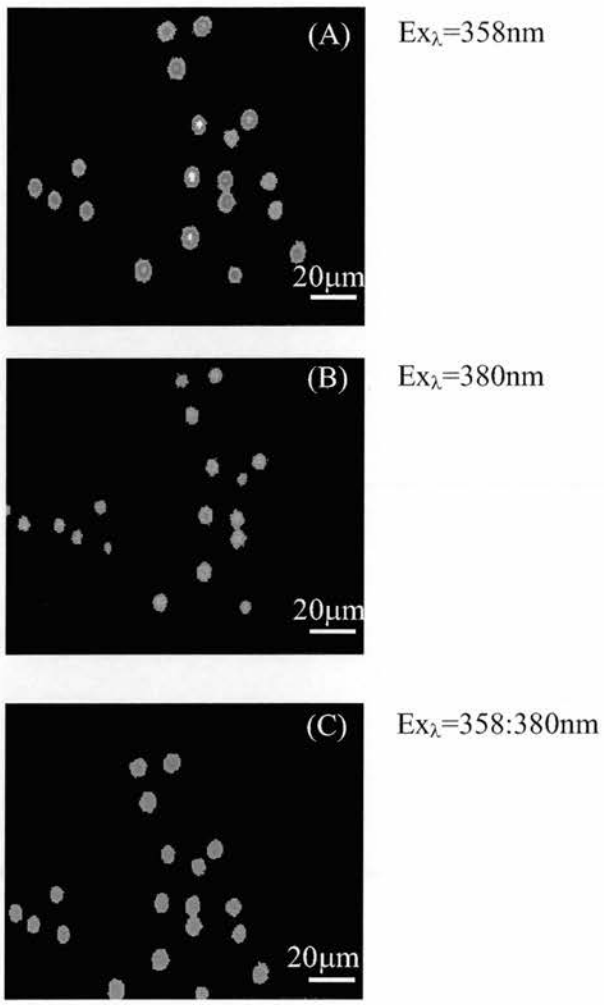


Figure 3.1. Fluorescent images of isolated bovine articular chondrocytes.

Chondrocytes were isolated from cartilage explants into 380mOsm.kg H_2O^{-1} DMEM and incubated with fura-2 AM (5µM; 37°C; 30mins). The cells were allowed to attach to glass coverslips (contained within steel rings), placed onto the stage a Nikon microscope and visualised through an x40 oil lens focused through the mid-plane. Images were acquired using a PTI ***Imagemaster™*** *system with the fura-2 excited alternately at 358nm (A; isobestic point, volume sensitive $[Ca^{2+}]_i$ insensitive) and 380nm (B; volume sensitive $[Ca^{2+}]_i$ sensitive) at 0.4Hz. A ratio image is also shown (C). The cells appeared rounded (as the above images are through a single optical plane the cells appear circular) with some heterogeneity in apparent cell size as seen by differences in cell diameter.*

3.2.2 Surface Rendered, Confocal Images of Chondrocytes.

To further illustrate the morphological differences between freshly-isolated and 2D cultured chondrocytes, images were acquired by Confocal Laser Scanning Microscopy (CLSM; see Materials and Methods) and 3D surface rendered images constructed. Briefly, using a high-powered Silicon Graphic Octane II UNIX workstation (California, US) running Imaris and VoxelShopPro software (Bitplane, Zurich, Switzerland), the 3D co-ordinate data acquired by CLSM was adjusted for fluorescent intensity and converted to a polygonal 3D surface image (Bush & Hall, 2001b, a, 2003). A computer generated light source was used to 'illuminate' the 3D rendered images to place further emphasis on cell shape.

Chondrocytes from bovine articular cartilage were visualised using CLSM microscopy following isolation into 380mOsm.kg H_2O^{-1} DMEM (a) within 5 hours, or (b) following 2D culture for up to three weeks as described (see Materials and Methods). Freshly isolated chondrocytes appeared spherical although there were noticeable differences in cell shape (some cells appeared less rounded) and this may relate to the cartilage zone from which the chondrocyte was derived (Fig. 3.2a). Conversely, with time in culture, there was a marked change in cell shape in the majority of the cells such that after ~3weeks the cells had flattened and spread out on the coverslip (Fig. 3.2b). There was no regularity between the shape of any of the cells and prior to cell passage the culture flask appeared ~80% confluent. These morphological data would strongly suggest that the cells may have begun to de-differentiate and that they may not represent the *in-vivo* chondrocytic phenotype.

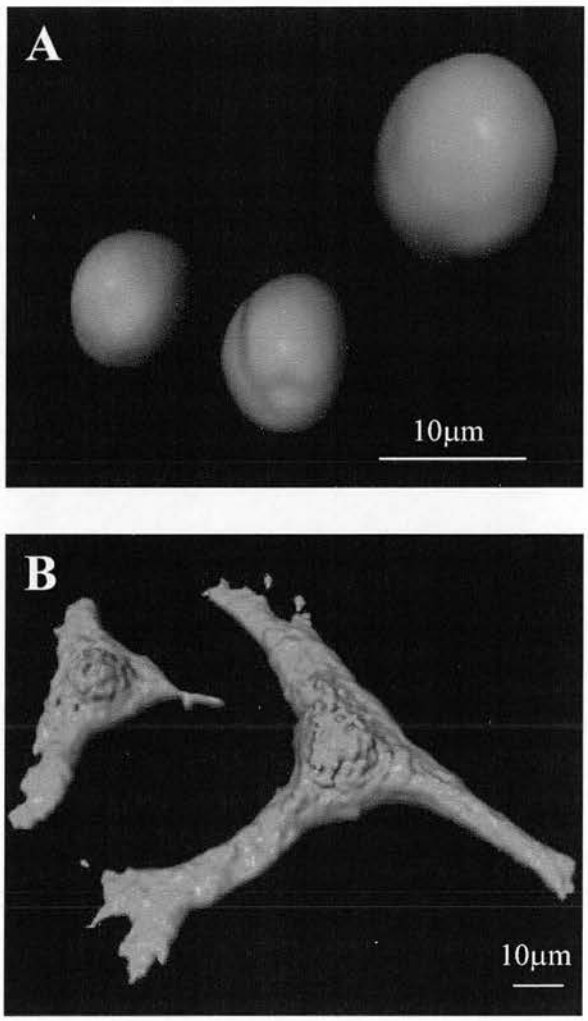


Figure 3.2. 3D surface rendered images of bovine articular chondrocytes.

Chondrocytes from bovine articular cartilage were isolated into 380mOsm.kg H_2O^{-1} DMEM see (Materials and Methods) and loaded with calcein (5μM; 30mins 37°C) 30mins prior to imaging. Aliquots of freshly-isolated cells were then visualised by confocal microscopy Zeiss Axioskop LSM510 upright confocal microscope system; 1μm increments), (A) immediately after isolation or (B) cultured as described (see Materials and Methods) for up to 2 weeks. After time in culture, the morphology of the chondrocytes changed from rounded appearance to a flatter, irregular shape. Scale bar represents 10μm. During analysis, an artificial computer-generated light source was added to further illustrate the morphological changes.

3.2.3 Changes in Volume of a Single Isolated Chondrocyte Following a Hypo-osmotic Challenge.

In a typical experiment, the resting fluorescence (V_{rest}) of freshly-isolated, fura-2 loaded chondrocyte was recorded under constant perfusion with the iso-osmotic 'control saline' (380mOsm.kg H_2O^{-1}) for 3mins and the fluorescence recorded. The perfusion solution was then switched to deliver a hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}), which resulted in a rapid fall in intracellular 358nm fluorescence (measured at 510nm emission) indicating an increase in cell volume (Fig. 3.3). The maximum increase in volume was estimated by the minimum fluorescence reading that occurred at ~1.5mins following the hypotonic challenge as shown (V_{max}). RVD was shown by the progressive increase of fluorescence over 10mins (V_{final}) following the osmotic challenge.

The hypo-osmotic challenge initiated a rise in $[Ca^{2+}]_i$ that appeared to correlate with the onset of RVD. The maximal rise in $[Ca^{2+}]_i$ occurred at ~1.5mins after the hypo-osmotic challenge and interestingly, this was approximately the same time are the maximal change in cell volume. $[Ca^{2+}]_i$ then started to return to basal levels during the remainder of the 10 minute experiment. At approximately 8.5mins, there appeared to a smaller rise in $[Ca^{2+}]_i$ (when compared to the maximal rise as a result of the osmotic challenge) and this could have been the result of a spontaneous $[Ca^{2+}]_i$ transient. In chondrocytes it has been reported that under 'resting' conditions there are $[Ca^{2+}]_i$ transient in approximately 6% of cells (Yellowley *et al.*, 1997; Yellowley *et al.*, 1999).

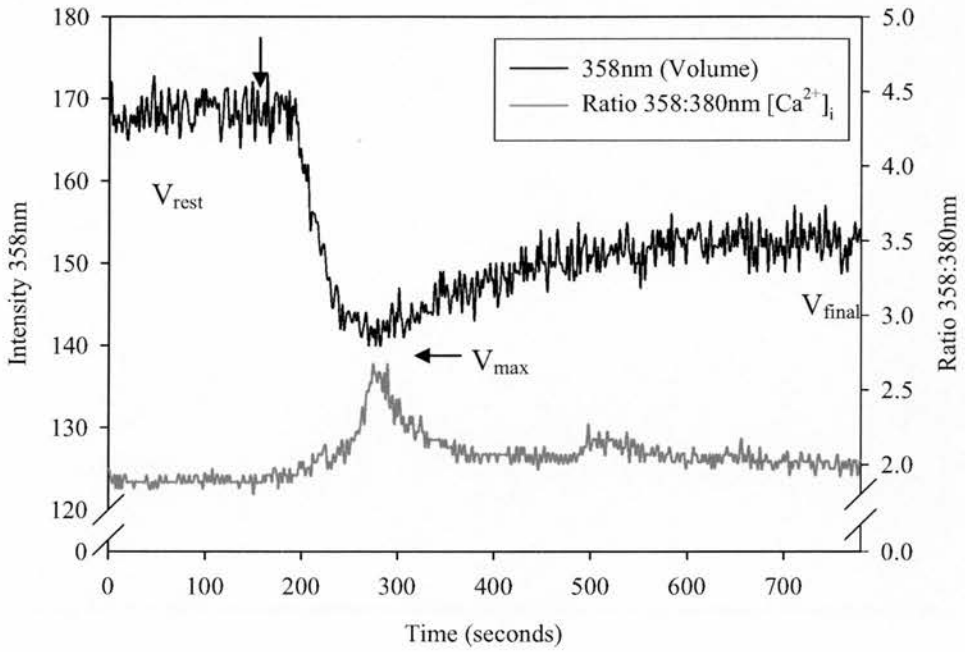


Figure 3.3. Regulatory volume decrease in a single chondrocyte following hypotonic challenge.

Freshly-isolated chondrocytes were incubated with fura-2 AM (5μM; 30mins 37°C) in DMEM (380mOsm.kg H_2O^{-1}) and excited at 358nm (isobestic point, volume-sensitive, calcium-insensitive wavelength) and emission measured at 510nm. Resting intensity (V_{rest}) was recorded for 3mins in an isotonic saline (380mOsm.kg H_2O^{-1}) and then a hypo-osmotic challenge (220mOsm.kg H_2O^{-1}; 43%) was applied by perfusion causing a rapid fall in intracellular 358nm fluorescence (shown by the black arrow). The maximum increase in volume was indicated by the minimum fluorescence after about 1.5mins as shown (V_{max}). RVD occurred as shown by the recovery of fluorescence where at the end of the experimental period (V_{final}) the cell had performed significant RVD. In response to the decrease in extracellular osmolality there was a transient rise in $[Ca^{2+}]_i$ that then return to basal levels during the remainder of the experiment (red line). Data are from one cell.

3.2.4 RVD by Freshly Isolated Chondrocytes Under Different Conditions.

The process of RVD was studied by fluorescent microscopy in freshly-isolated chondrocytes in response to a 43% hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}). To further elucidate the mechanism of RVD, salines consisting of (a) the 'control saline' (1mM Ca^{2+}), (b) 'Ca^{2+}-free saline' (no added Ca^{2+} with 2mM EGTA), (c) 'high Ca^{2+}-saline' (Ca^{2+} = 20mM), (d) 'Gd^{3+}-saline' (Gd^{3+} = 100μM; an inhibitor of chondrocyte stretch-sensitive ion channels; Wright *et al.*, 1996), or (e) 'Dantrolene-saline' (20μM; inhibitor of $[Ca^{2+}]_i$ release; Frandsen & Schousboe, 1992; Charles *et al.*, 1993; Alvarez-Vega *et al.*, 2001), (f) 'REV 5901-saline' (50μM; inhibitor of LTD_4; Aharony *et al.*, 1986; Choi *et al.*, 1986) were used. Data were recorded for 2-3 mins under iso-osmotic conditions and then the saline switched to deliver a saline of 220mOsm.kg H_2O^{-1}. Data were subsequently recorded for 10mins.

In response to the decrease in extracellular osmolality, there was a fall in the 358nm emission indicating an increase in cell volume. When comparing the maximal volume change (V_{max}) for all experimental conditions, it was found that there was no significant difference (Student's t-test; $p>0.05$). Some chondrocytes subsequently underwent RVD within the 10min experimental period and for all data sets, RVD proceeded linearly with an r^2 value equal or greater than 0.91 (as calculated by linear regression; see Materials and Methods).

In the control (1mM Ca^{2+}) saline, 59.53 +/- 7.19 % of cells exhibited greater than 50% RVD and this was significantly decreased (Student's t-test; $p<0.05$) by the removal of extracellular calcium plus the addition of 2mM EGTA. Conversely, the addition of gadolinium (an inhibitor of stretch activated ion channels; (Wright *et al.*, 1996), or Dantrolene (an inhibitor of intracellular calcium release (Charles *et al.*, 1993) had no effect ($p>0.05$) on the number of cell showing RVD. The process of RVD was significantly reduced ($p<0.01$) by REV 5901 (50μM) indicating that it was a useful inhibitor of chondrocyte RVD (Fig. 3.4).

When comparing the rate of RVD in cells that did respond, there was no significant difference for any of the experimental groups ($p>0.05$) when compared to the '1mM control saline'. The rate in the EGTA saline was borderline to being significantly slower ($p = 0.0512$) indicating that the removal of extracellular calcium does attenuate the response. It is possible that a greater concentration of EGTA may further influence the rate of RVD as it is possible that there may be a small amount of calcium (from trace contamination) in the experimental saline. Values for the $t_{½}$ for RVD of each experimental group are given in (Table 3.1).

When reviewing the data shown in Table 3.1, it is noticeable that there is a large heterogeneity within the data sets as indicated by the s.e.m. All these experiments were performed on freshly-isolated chondrocytes excised from full-depth cartilage explants and therefore encompass chondrocytes from all the cartilage zones. This, in conjunction with the fact that analysis is performed per joint and not by cell (therefore including the

heterogeneity between animals; see Materials and Methods), provides a more accurate representation of the parent population.

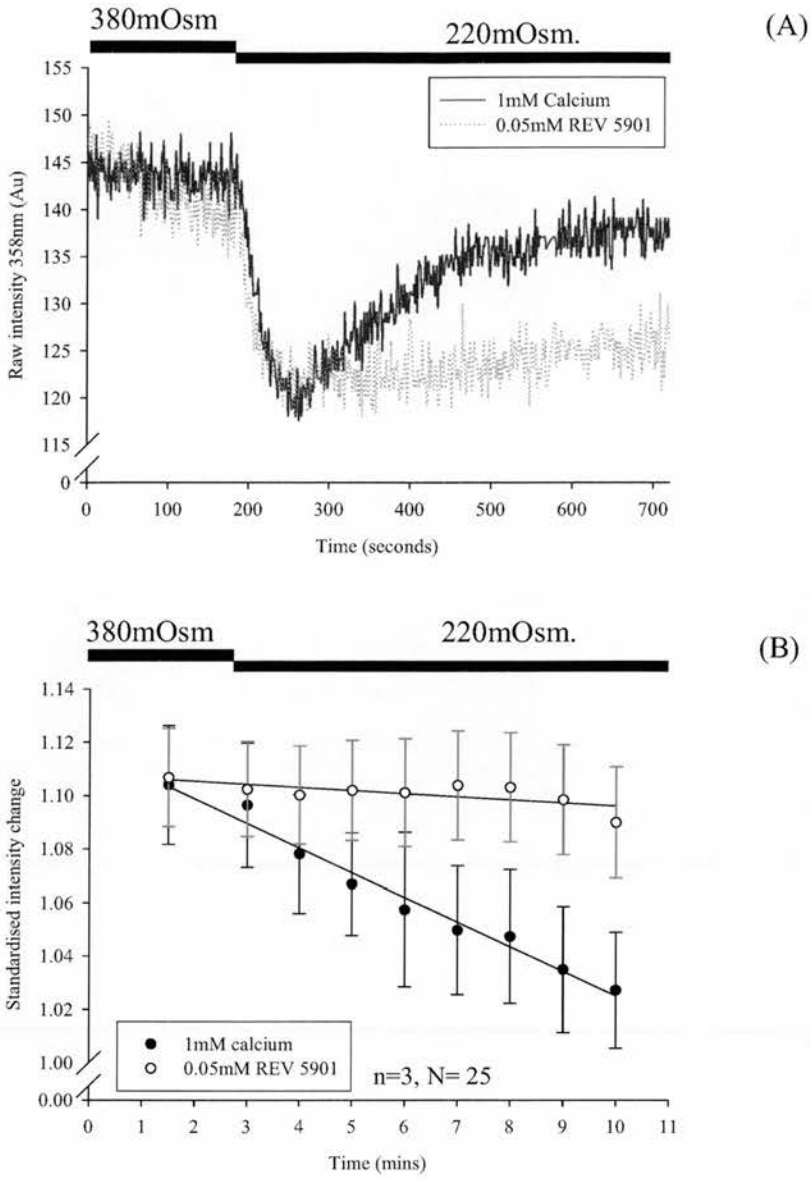


Figure 3. 4. Inhibition of RVD by REV 5901 in freshly-isolated chondrocytes.

Chondrocytes were isolated from full-depth bovine articular cartilage explants and RVD studied by fluorescent microscopy in response to a 43% hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}). (A) Example traces of a chondrocyte volume regulating by RVD (dark blue line) and one in which RVD is inhibited by REV 5901 (light blue line). (B) Pooled RVD data from one joint showing the inhibition of RVD by REV 5901. Data are expressed as mean ± s.e.m with the number of joints shown as n and the number of cells as [N]. In this example, the s.e.m was calculated using the number of cells and not the number of joints (see Materials and Methods).

Experimental condition	Cell total (No. joints, [cell No])	% RVD	t½ (mins)
Control (1mM Ca^{2+})	11 [254]	59.53 ± 7.19	6.67 ± 0.63
EGTA (2mM)	6 [171]	22.96 ± 5.11	9.08 ± 3.71
High Ca^{2+} (20mM)	3 [83]	50.20 ± 6.98	6.88 ± 0.42
Gd^{3+} (100μM)	6 [177]	45.54 ± 4.89	8.13 ± 0.94
REV 5901 (50μM)	3 [25]	0.00 ± 0.00	N/S
NDGA[3] (75μM)	3 [55]	4.00 ± 2.5	N/S
Dantrolene (30μM)	3 [155]	50.52 ± 14.19	4.99 ± 0.84

Table 3. 1. RVD by freshly-isolated chondrocytes under different conditions.

RVD was studied in freshly-isolated chondrocytes incubated with fura-2 AM (5μM; 30mins) in response to a 43% hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}). In response to the osmotic challenge some cells from all experimental groups responded with RVD, although the number of cell responding was significantly decreased (Student's-test; $p<0.05$) by the removal of extracellular calcium (plus the addition of EGTA). RVD was significantly inhibited by both REV 5901 ($p<0.001$) and NDGA ($p<0.001$) indicating that these are good inhibitors of the response. Conversely, the inhibition of stretch activated channels by gadolinium or the inhibition of intracellular calcium store release had no effect ($p>0.05$). Data are expressed as mean ± s.e.m (s.e.m calculated inbeteen joints and not cells; see Materials and Methods). The number of joints and cells are shown in the 'Cell total' column with 'n' as the number of joints and [N] as the number of cells.

[3] Experiment data from Dr A.C. Hall

3.2.5 RVD by Cultured Chondrocytes Under Different Conditions

The data presented previously have shown that freshly-isolated chondrocytes have the capacity for RVD and that the response is attenuated by the removal of extracellular calcium but was not inhibited by gadolinium or dantrolene (Table 3.1). As many of the experiments studying the chondrocyte response to mechano-perturbation are performed on cultured cells (see section 1.6.0), RVD was studied in 2D cultured chondrocytes. As with the freshly-isolated chondrocytes, to further elucidate the mechanism of RVD, salines consisting of (a) the 'control saline' (1mM Ca^{2+}), (b) 'Gd^{3+}-saline' (Gd^{3+} = 100μM; an inhibitor of chondrocyte stretch-sensitive ion channels; (Wright *et al.*, 1996), or (c) 'REV 5901-saline' (50μM; inhibitor of Leukotrine LTD_4 receptor Aharony *et al.*, 1986; Bush & Hall, 2001b) were used[4].

Chondrocytes were isolated from full depth cartilage explants and cultured as previously described (see Materials and Methods). Eighteen hours prior to an experiment, cells were passaged onto sterile (autoclaved) 22mm coverslips and maintained in culture until required. In response to the decrease in extracellular osmolality, it was noted that unlike the freshly-isolated chondrocytes there was an increase in 358nm fluorescent intensity as opposed to the expected decrease. It has been commented that uniform cell swelling in chondrocytes requires rounded cells (Yellowley *et al.*, 2002) and this may explain the fluorescent increase. There was no significant difference ($p>0.05$) when comparing the extent of cell swelling between any of the experimental groups significantly with changes of fluorescence of 12.9 ± 2.5%,

[4] It this study the removal of extracellular calcium was also tried but due to time constraints it was not possible to get this to work.

11.6 ± 0.4% and 12.1 ± 2.4% ('control saline', 'Gd^{3+}-saline' & 'REV 5901-saline') respectively (Table 3.2).

As seen in the freshly-isolated chondrocytes, a pre-incubation with gadolinium had no effect on the percentage of cells showing RVD (41.5 ± 7.3% compared to 41.9 ± 1.15%). Conversely, the pre-incubation with REV 5901 significantly decreased (Student's t-test; $p<0.0001$) the number of cells responding. Interestingly, the inhibitory effect was not as marked as that observed in freshly-isolated chondrocytes with 13.7 ± 2.4% still showing RVD compared to 0.0 ± 0.0% in freshly-isolated cells (Table 3.2).

Experimental condition	Cell total	% RVD	t½ (mins)
Control (1mM Ca^{2+})	3,[110]	41.92 ± 1.10	7.86 ± 1.36
Gd^{3+} (100μM)	4,[179]	41.51 ± 7.29	6.51 ± 1.21
REV 5901 (50μM)	3,[154]	13.74 ± 2.36	8.01 ± 2.01

Table 3. 2. RVD by 2D cultured chondrocytes under different conditions.

Chondrocytes were isolated from full depth articular cartilage explants and cultured on plastic for 2 weeks. Cells were then passaged onto sterile 22mm coverslips and incubated with fura-2 (5μM; 30mins) for experimentation. RVD was studied in response to a 43% hypo-osmotic challenge by perfusion. 2D cultured chondrocytes were able to volume regulate by RVD and this was not inhibited by a pre-incubation with gadolinium (inhibitor of stretch-activation ion channels). Conversely, and as seen in freshly-isolated chondrocytes, REV 5901 significantly inhibited the response (Student's t-test; $p<0.0001$) although to a lesser extent. Data are expressed as mean ± s.e.m. The number of joints is shown as n and the number of cells as [N].

3.2.6 Changes in $[Ca^{2+}]_i$ in Freshly–Isolated Chondrocytes in Response to a Hypo-osmotic Challenge.

As shown in Figure 3.1, a decrease in extracellular osmolality initiated a rise in $[Ca^{2+}]_i$ in some cells. To examine the $[Ca^{2+}]_i$ rise of freshly-isolated and cultured chondrocytes during hypo-osmotic challenge, selected agents (as previously used to study RVD) were tested (Table 3.1). For freshly-isolated chondrocytes in 'control saline' conditions, 61.8 ± 9.1% of cells responded to the hypotonic shock with a rise in $[Ca^{2+}]_i$ which was not significantly different ($p>0.05$) in the presence of Gd^{3+} but significantly ($p<0.05$) inhibited in 'Ca^{2+}-free saline' where only 9.6 ± 4.8 % responded. Interestingly in chondrocytes treated with REV 5901, there was no inhibition of the number of cells showing a rise in $[Ca^{2+}]_i$. Dantrolene, also failed to decrease the percentage of cells showing a rise in $[Ca^{2+}]_i$ (76.01 ± 14.9 % of cells responded) and this would imply that the rise is not mediated by a release from dantrolene-sensitive intracellular calcium stores.

There did not appear to be a correlation with the rise in $[Ca^{2+}]_i$ and the RVD response as shown Figure 3.5. In some cells there was a rise in $[Ca^{2+}]_i$ and RVD (Fig. 3.5a) whereas other cells underwent RVD with no apparent change in $[Ca^{2+}]_i$ (Fig. 3.5b). Furthermore, in some cells, there was a rise in $[Ca^{2+}]_i$ and no volume regulation (Fig. 3.5) and in others there was neither a rise in $[Ca^{2+}]_i$ or RVD (Fig.3.5d). This difference in response is mostly likely attributed to the heterogeneous cell population being studied and may relate to the zone from which the cells were derived. It further shows the heterogeneity of the chondrocyte response and the problems of working with primary cells.

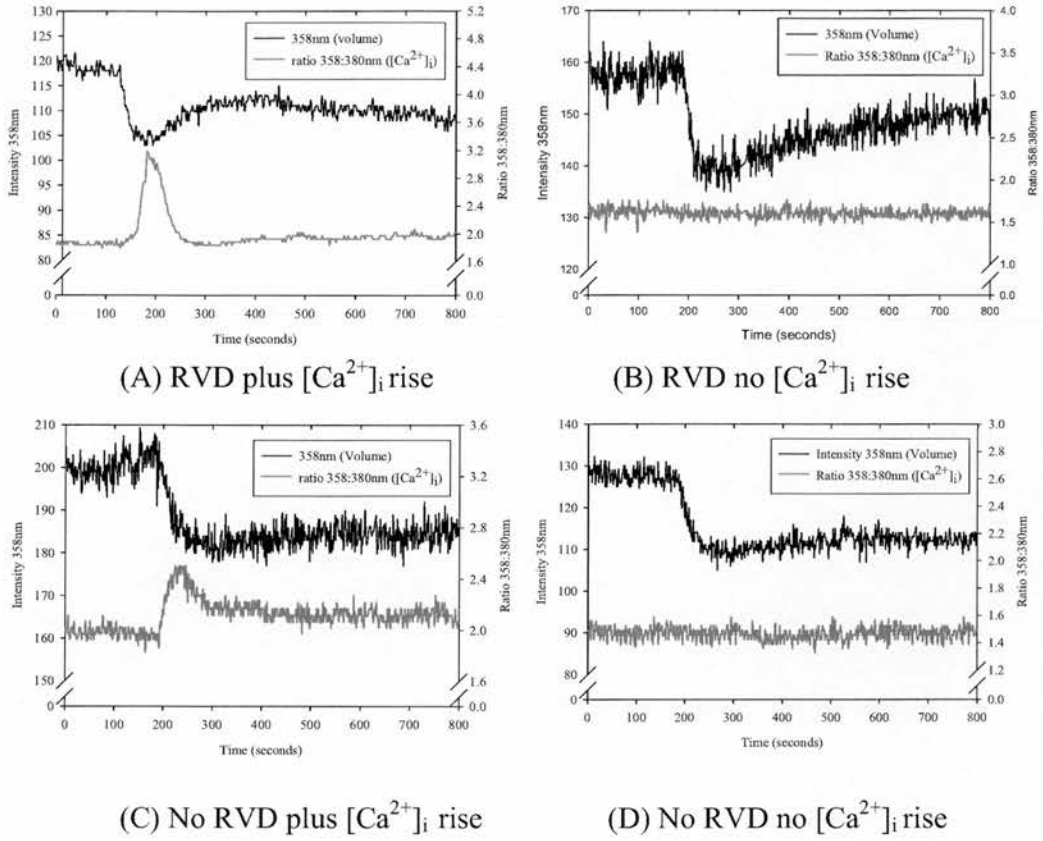


Figure 3.5. Heterogeneity of the changes in $[Ca^{2+}]_i$ isolated bovine articular chondrocytes.

Chondrocytes were isolated into 380mOsm.kg H_2O^{-1} DMEM, incubated with fura-2 AM (5μM, 37°C; 30mins) and visualised by fluorescent microscopy as previously described (see Materials and Methods). Chondrocytes were perfused with an iso-osmotic saline for 2mins and then a 43 % hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) was applied by perfusion. In response to the decrease in extracellular osmolality, some chondrocytes performed RVD (shown as the black lines) and in some instances there was a rise in $[Ca^{2+}]_i$ (shown as the red lines; Fig A & B). Conversely, other chondrocytes did not perform RVD although there was still a rise in $[Ca^{2+}]_i$ in some cells (Fig (C & D). These data further show the heterogeneous response of the chondrocyte population where there did not appear to be a correlation between changes in $[Ca^{2+}]_i$ and the capacity for RVD. Furthermore, the magnitude of the $[Ca^{2+}]_i$ rise did not appear to be the same between cells. Data are expressed as raw fluorescence traces, n=4 joints, N=4 cells.

Using the experimental saline with no added calcium plus 2mM EGTA, the rise in $[Ca^{2+}]_i$ in response to a decrease in extracellular osmolality was inhibited in most cells. In fact, there was a decrease in the 358:380nm ratio that would suggest that $[Ca^{2+}]_i$ had decreased. This is most likely a result of the influx of EGTA through a channel that had opened in response to the osmotic decrease; potentially the same channel that mediated the calcium influx, although it is also possible that there was a change in the efflux of calcium that was not compensated for via a calcium influx due to the lack of calcium. Despite this, in some cells there was still a rise in $[Ca^{2+}]_i$ and this was most likely from a release for dantrolene-insensitive stores. In the 1mM calcium saline, the rise in $[Ca^{2+}]_i$ occurred near to the time of maximal cell swelling, whereas in the 2mM EGTA saline the maximal intracellular calcium rise occurred later. This may imply that in response to a decrease in osmolality there are two pathways for the $[Ca^{2+}]_i$ rise, one mediated via an influx and the second from non-calcium induced calcium release stores (Fig 3.6). Interestingly, the cells that did show RVD in the 2mM EGTA saline, none exhibited a rise in $[Ca^{2+}]_i$ greater than 5% above basal levels.

In cultured chondrocytes, in response to a 43% hypo-osmotic challenge, 44.8 ± 5.5% of chondrocytes responded with a rise in $[Ca^{2+}]_i$ that was also significantly inhibited (Student's t-test; $p<0.05$) in 'Ca^{2+}-free saline' (preliminary data). However unlike freshly-isolated chondrocytes, in the presence of Gd^{3+} (100μM) the $[Ca^{2+}]_i$ rise was significantly reduced ($p<0.05$) with only 19.5 ± 4.5% of cells responding. Thus this would appear to indicate that during culture there was a change in the expression or regulation of stretch sensitive, $[Ca^{2+}]_i$ channels.

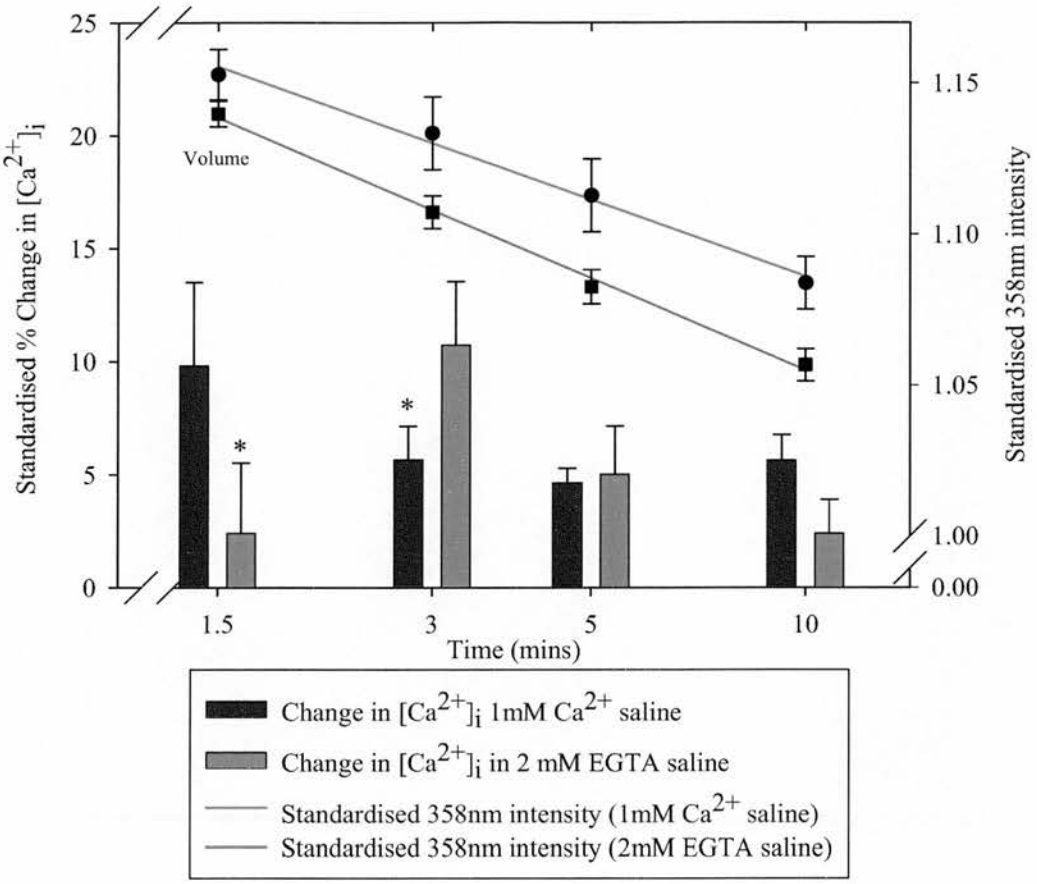


Figure 3.6. RVD and changes in $[Ca^{2+}]_i$ in response to a 43% hypo-osmotic challenge.

Chondrocytes were isolated from full depth bovine articular cartilage explants into 380mOsm.kg H_2O^{-1} DMEM, incubated with fura-2 AM (30mins; 5μM) and RVD measured in response to 43% hypo-osmotic challenge. RVD was recorded in (a) 1mM calcium saline (Blue) and (b) 2mM EGTA saline (red). Changes in $[Ca^{2+}]_i$ were also calculated as a percentage change over 'resting levels' (see Materials and Methods). In response to the osmotic decrease, there was an increase in cell volume with no significant difference in V_{max} between the two experimental groups. RVD then proceeded linearly with no significant difference in rate. There was a rise in $[Ca^{2+}]_i$ at maximal cell swelling that was significantly inhibited by the removal of extracellular calcium. At 3mins, there was a rise in $[Ca^{2+}]_i$ in cells in the 2mM EGTA media. This may have been the result of release from intracellular calcium stores. Data are shown as mean ± s.e.m with n=9 joints, 156 cells.

3.2.7 Mn^{2+} Quenching Suggests That the Rise in $[Ca^{2+}]_i$ is Due to Influx.

As previous shown (section 3.2.6), a decrease in extracellular osmolality initiated a rise in $[Ca^{2+}]_i$ in some chondrocytes that during the time course of the experiment returned towards the basal levels. One possible source of calcium is from the extracellular environment and the hypo-osmotic stimulus initiates a calcium influx. It has been shown in response to fluid flow and direct mechanical stimulation, the rise in $[Ca^{2+}]_i$ was inhibited by the removal of $[Ca^{2+}]_o$ thus supporting the theory of calcium influx in response to mechanical stimulation (Guilak *et al.*, 1999b; Yellowley *et al.*, 2000; Edlich *et al.*, 2001). Therefore, using the Mn^{2+} quenching technique (where Mn^{2+} influx binds fura-2 and quenches fluorescence (Chiavaroli *et al.*, 1994; Plieth *et al.*, 1998; Fellner & Arendshorst, 2000), the role for calcium entry was studied in response to a decrease in extracellular osmolality.

Chondrocytes were perfused with a 1mM Mn^{2+} (380mOsm.kg H_2O^{-1}) saline for two mins and the saline switched to deliver a hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}; hypo-osmotic saline also containing 1mM Mn^{2+}). Data were recorded for the subsequent 10mins. In response to hypo-osmotic challenge, there was a decrease in the 358nm fluorescence (measured at the 510nm emission) of all cells indicating an increase in cell volume. For some cells the 358nm signal continued to decrease indicating that the fura-2 was being quenched (Fig. 3.7). Conversely, in other cells the 358nm fluorescence stabilised at the minimum cell volume. These data further shows the heterogeneity of the chondrocyte population, as not all cells showed a rise in

$[Ca^{2+}]_i$ in response to a hypo-osmotic challenge and therefore not all cells will quench. There was a noticeable lack of RVD in most cells, and this may have been because in a calcium free saline the RVD response is attenuated (section 3.2.4). Interestingly, a few cells did exhibited RVD (~14%) and this would imply that the response was independent of a rise in $[Ca^{2+}]_i$ else the 358nm fluorescent signal would have been quenched due to the influx of Mn^{2+} into the cell.

To summarise, this data supports the notion that the rise in $[Ca^{2+}]_i$ in some chondrocytes during hypo-osmotic challenge arises from Ca^{2+} influx. This data does not rule out the involvement of calcium release from intracellular stores and recently, it has been shown in cultured BACs that a pre-incubation with thapsigargin attenuates an $[Ca^{2+}]_i$ rise (Yellowley *et al.*, 2002) in response to an osmotic challenge.

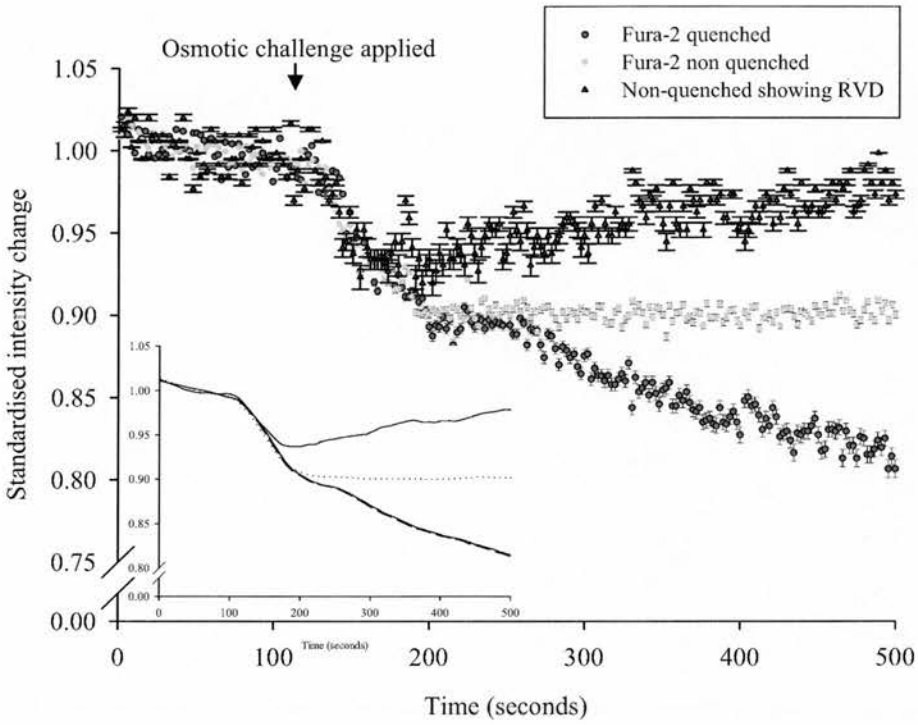


Figure 3.7. Mn^{2+} quenching of fura-2 in response to a hypo-osmotic challenge.

Chondrocytes were isolated and incubated with fura-2 as previously described (see Materials and Methods). To show the differences between quenching and non-quenching cells that can account for the differences in initial 'resting' fluorescence, data were standardised to the fluorescence recorded during the 'resting period'. Therefore, a value of 1.0 is that in an iso-osmotic saline and values below this indicate cell swelling. In response to a decrease in extracellular osmolality (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}), there was a fall in 358nm fluorescence indicating an increase in cell volume. In some cells there was a continued decrease in fluorescence that would imply that the fura-2 signal was being quenched by the Mn^{2+}. Conversely, in other cells there was no further change in the 358nm emission. A few cells performed RVD as seen by the increase in the standard intensity back towards 1.0. Interestingly, the extent of cell swelling appeared less but the number of cells showing RVD is too small to draw any decisive conclusions. Insert: To further illustrate the differences in the three data traces, data were 'smoothed' using Jandel Sigma Plot function. These data suggest that in some cells, in response to the hypo-osmotic challenge the rise in $[Ca^{2+}]_i$ is mediated by an influx across the plasma membrane. Data are shown as standardised fluorescent traces with the s.e.m calculated for each point. N = 2 joints, N=44 cells.

3.2.8 The Rise in $[Ca^{2+}]_i$ did not Appear to Correlate with the Capacity for RVD.

RVD was recorded in freshly-isolated chondrocytes in response to a 43% hypo-osmotic challenge by perfusion. Changes in volume and $[Ca^{2+}]_i$ were recorded and the chondrocytes grouped as either 'responding' (cells showing greater than 50% RVD) and 'non-responding' (cells showing less than 50% RVD; see Materials and Methods).

For both groups, there was no significant difference ($p > 0.05$) in the extent of maximal swelling (V_{max}), as shown by the change in 358nm intensity but there was a significant difference (Student's t-test $p<0.001$) at 10mins post hypo-osmotic challenge (Fig.3.8). This difference at 10mins (V_{final}) confirmed that the groups were separate and distinct from each other with respect to volume regulation. The corresponding changes in $[Ca^{2+}]_i$ were plotted and are shown (Fig. 3.8b). In the 'responding' and 'non-responding' groups there was an identical rise in $[Ca^{2+}]_i$ in response to the hypo-osmotic challenge with no significant difference ($p>0.05$) in the maximal rise, duration of the response or the recovery during the remainder of the experimental period. Furthermore, when correlating the percentage of chondrocytes volume regulating with a rise in $[Ca^{2+}]_i$ in a 1mM calcium 'control saline', it was found that 41% of cells exhibited RVD with a rise in $[Ca^{2+}]_i$, 25% exhibited RVD with no rise in $[Ca^{2+}]_i$, 26% of cells exhibited no RVD but still a rise in $[Ca^{2+}]_i$ and 8% exhibited neither a rise in $[Ca^{2+}]_i$ or RVD (figures based on whole cell population and therefore not possible to calculate an error; N=107 cells).

These data strongly suggest that a rise in $[Ca^{2+}]_i$ is not essential for chondrocyte RVD and that the $[Ca^{2+}]_i$ rise may be an epiphenomenon associated with cell swelling. Alternatively, the $[Ca^{2+}]_i$ may be involved in another chondrocyte response related to changes in volume but not in its' regulation. It is possible that due to the heterogeneous nature of the chondrocytes populations studied, some cells require a rise in $[Ca^{2+}]_i$ to mediate the RVD response whereas other cells do not.

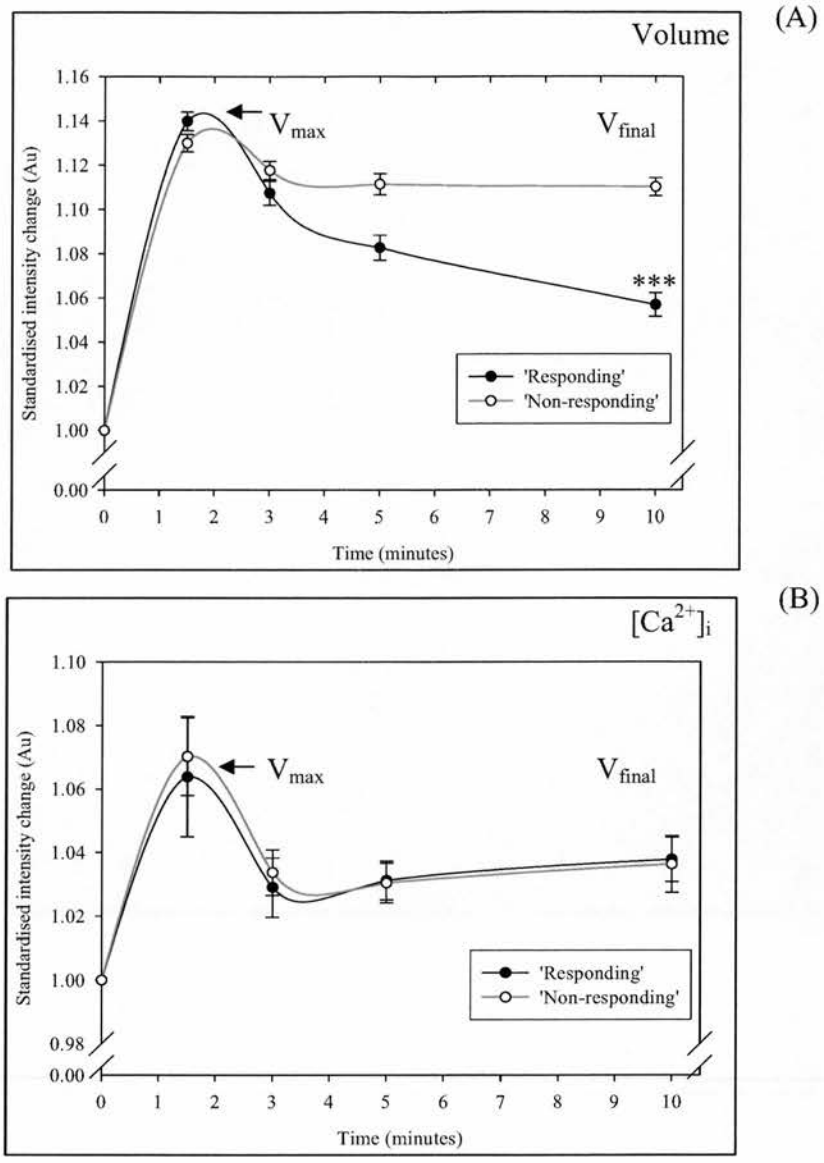


Figure 3. 8. The rise in $[Ca^{2+}]_i$ does not correlate with the capacity for RVD.

RVD was studied in freshly-isolated chondrocytes in response to a 43% hypo-osmotic challenge. Changes in volume and $[Ca^{2+}]_i$ were recorded and the chondrocytes grouped as either 'responding' (cells showing greater than 50% RVD) and 'non-responding' (cells showing less than 50% RVD; see Materials and Methods). (A) There was no significant difference in the extent of cell swelling (V_{max}) between 'responding' and 'non-responding' groups. At the end of the experimental period, there was a significant difference (Student's t-test; $p<0.001$) indicating the difference between the two experimental groups. (B) When comparing the changes in $[Ca^{2+}]_i$ for both groups there was no significant difference ($p>0.05$). These data would imply that the $[Ca^{2+}]_i$ rise may not be a mediator in the RVD response. Data are expressed as standardised intensity changes with the mean and s.e.m shown. n= 5 joints, N= 120 cells.

3.2.9 A Change in Gd^{3+} Sensitivity of Over Time Occurs in Culture.

It was shown in section 3.2.6 that a decrease in extracellular osmolality initiated a transient rise in $[Ca^{2+}]_i$ that subsequently returned towards basal levels during the 10min experimental period. In freshly-isolated chondrocytes, a pre-incubation with gadolinium did not inhibit the $[Ca^{2+}]_i$ rise and therefore it seems that it is unlikely to be mediated by gadolinium-sensitive and consequently a stretch-activated channel (Table 3.1). Conversely, after 3 weeks in 2D culture (see Materials and Methods) and under the same experimental conditions, the pre-incubation with gadolinium inhibited the rise in $[Ca^{2+}]_i$ (Fig. 3.9). In freshly-isolatedand 2D cultured chondrocytes, the removal of extracellular calcium plus the addition of 2mM EGTA significantly inhibited the response ($p<0.0001$).

These data imply that following culture, stretch-activated ion channels are involved in mediating the rise in $[Ca^{2+}]_i$ and that for some yet to be determined reason the expression of stretch activated ion channels changes during culture. The $[Ca^{2+}]_i$ rise is a result of a calcium influx either through a stretch-sensitive calcium channel or mediated via a stretch-sensitive signalling pathway.

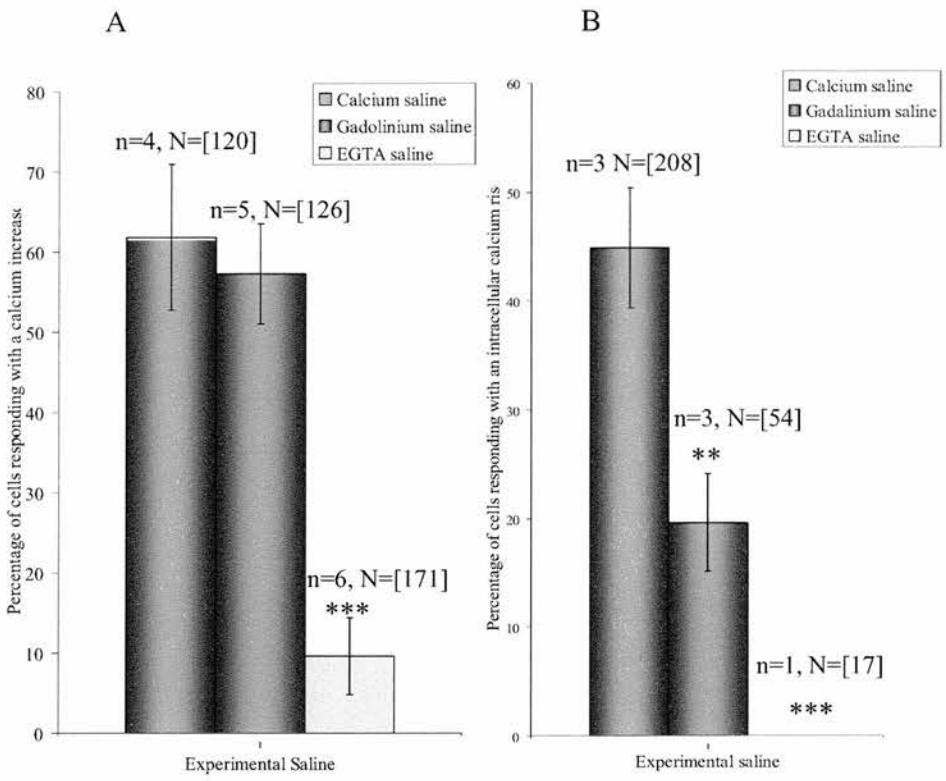


Figure 3.9. A change in gadolinium sensitivity occurs during 2D culture.

Changes in $[Ca^{2+}]_i$ calcium in response to a decrease in extracellular osmolality were studied in (A) freshly-isolated chondrocytes and (B) 2D cultured chondrocytes. (A) In control '1mM calcium saline', the majority of cells responded with a rise in $[Ca^{2+}]_i$ and this was not attenuated by a pre-incubation with gadolinium (100μM) but completely inhibited by the removal of all extracellular calcium plus the addition of EGTA. Conversely, in 2D cultured chondrocytes, less cells seems to respond with a rise in $[Ca^{2+}]_i$ and unlike freshly-isolated cells the number of cell responding was significantly decreased (Student's t-test; $p<0.01$) by a pre-incubation with gadolinium. As observed in freshly-isolated cells, preliminary EGTA data show that the response is inhibited by the removal of extracellular calcium. Data are expressed as the percentage of cell responding with the mean and s.e.m shown. n= number of joints, N= number of cells.

3.2.10 Lack of Response of Chondrocyte $[Ca^{2+}]_i$ to a High K^+ Saline.

It was conceivable that the rise in $[Ca^{2+}]_i$ which occurred in response to a hypo-osmotic challenge was mediated by a change in membrane potential and the subsequent activation of voltage-activated Ca^{2+} channels (VACC). To test this, freshly-isolated and cultured chondrocytes were perfused with a 'high K^+ saline' and changes in $[Ca^{2+}]_i$ recorded using fura-2. In both chondrocyte preparations, there was no significant difference (Student's t-test; $p>0.05$) in the 358:380nm ratio in response to the 'high K^+ saline (Table 3.3). Interestingly, after long term culture (4 passages) there was still no $[Ca^{2+}]_i$ response to the 'high K^+ saline'. This would seem to indicate that de-differentiation (assumed to have occurred over this period; Zaucke *et al.*, 2001) does not induce the expression of voltage-activated calcium channels in bovine articular chondrocytes.

Experimental saline	Freshly isolated chondrocytes	Cultured, 'flattened' chondrocytes	Passage 4 2D cultured chondrocytes
Ratio	340:380nm	340:380nm	358:380nm
Control 1mM Ca^{2+}	0.695 ± 0.100	0.886 ± 0.085	1.403 ± 0.132
High K^+ saline 120mM K^+	0.687 ± 0.102	0.885 ± 0.087	1.397 ± 0.133
Cell total	n= 6 N= [197]	n=3 N=[59]	n=3 N=[80]

Table 3.3. Lack of response of chondrocyte $[Ca^{2+}]_i$ to a high K^+ saline.

Chondrocytes were isolated form bovine articular cartilage and either experimented on within 5 hours or placed into long-term culture. Prior to use, cells were incubated with fura-2 AM (5μM; 30mins; 37°C) and then imaged by fluorescent microscopy. A resting ratio was recorded for 3 mins in control saline and then the saline switch to deliver a high K^+ pulse. There was no significant difference (Student's t-test; $p<0.05$) in $[Ca^{2+}]_i$ recorded between resting and high K^+ salines. Both periods of 2 weeks and longer term culture had no effect on the lack of an $[Ca^{2+}]_i$ response. These data would imply that both freshly-isolated and 2D cultured chondrocytes do not express potential-sensitive calcium channels. Data are expressed as mean ± s.e.m. n= number of joints, N= number of cells.

3.2.10.1 Depolarisation of AtT20 cells using a High k^+ Saline.

To ensure the method of depolarisation was suitable for the study of $[Ca^{2+}]_i$, AtT20 cells (a gift from Dr M. J. Shipston and Dr L. Tian) were used as a control. AtT20 cells were passaged (as previously described) and plated onto sterile (autoclaved 22mm) coverslips. Cells were then incubated with fura 2 (5μM; 37°C; 30mins) and imaged as previously described (see Materials and Methods). Cells were perfused with a 1mM calcium saline (280mOsm.kg H_2O^{-1}; not 380mOsm.kg H_2O^{-1} as the prevailing osmolality for AtT20 cells is 280mOsm.kg H_2O^{-1}) for 60 seconds and the saline changed to a high K^+ for the remainder of the experiment.

In response to the high K^+ saline there was a rise in $[Ca^{2+}]_i$ as shown by an increase in the 340:380nm ratio (Fig. 3.10a). Intracellular calcium then began to return towards basal levels during the remainder of the experiment. When examining the individual emission at 510nm of the excitation wavelengths of the 340nm and 380nm it was possible to observe the 'free' and 'bound' calcium response for each (Fig. 3.10b). When there is a rise in $[Ca^{2+}]_i$, the fluorescent intensity of the 340nm signal increased as there was more calcium 'bound' to the fura-2 increasing the fluorescent emission (recorded at 510nm) for that particular wavelength. Conversely, there was a decrease in the 380nm signal (emission recorded at 510nm) as there was less 'free' calcium available. These data would imply that the method of a high K^+ saline was suitable to test for the presence of VACC activity.

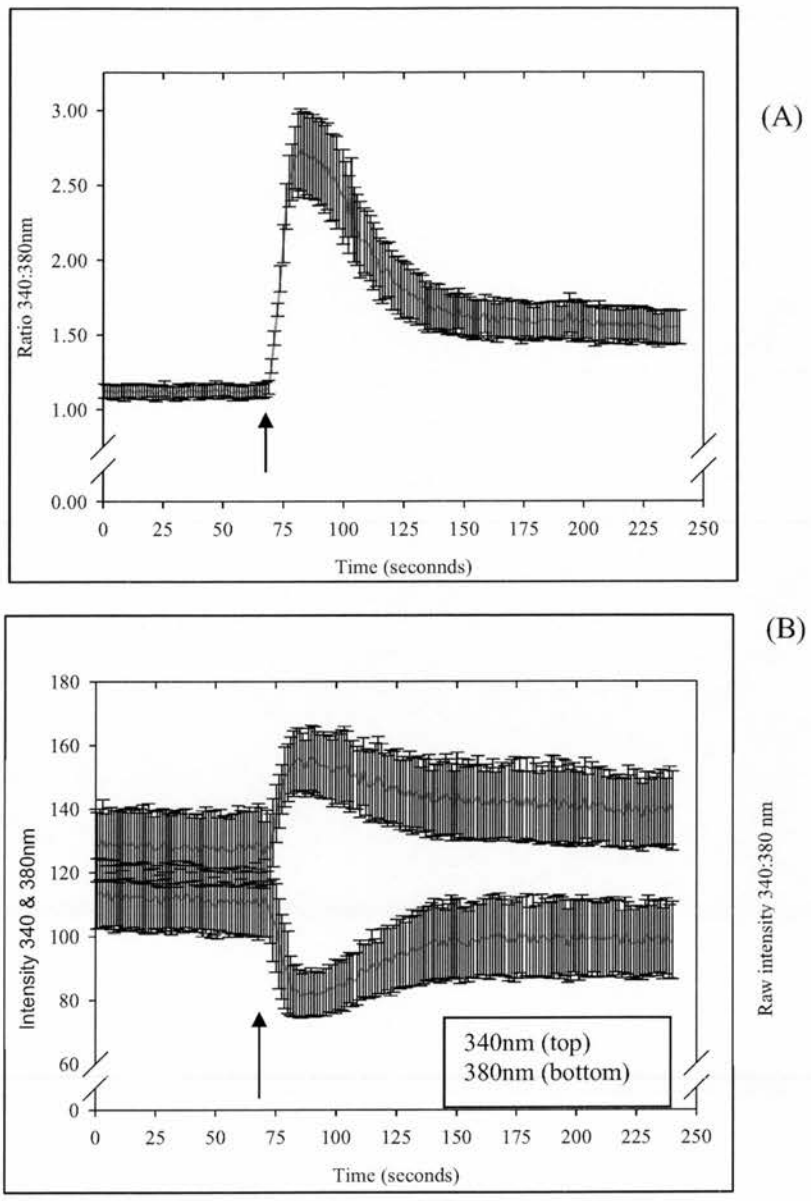


Figure 3.10. Changes in $[Ca^{2+}]_i$ in AtT20 cells during perfusion with a high K^+ saline.

AtT20 cells were passaged onto sterile glass coverslips, incubated with fura-2 AM (5μM; 37°C; 30mins) and visualised by fluorescent microscopy. Cells were perfused with a control (1mM calcium) saline for 60secs and then the saline was then changed to deliver a high K^+ saline (for composition see Materials and Methods). In response to the high K^+ saline there was a rise in $[Ca^{2+}]_i$ (as shown by the 340:380nm ratio) that subsequently began to return to basal levels (a). When examining each individual wavelength, the 340nm increased in fluorescent intensity and the 380nm decreased (b) in response to the high K^+ saline. Data are expressed as raw fluorescent ratio traces and an arrow shows changes in saline. N=6 cells.

3.2.10.2 The Effect of a high K^+ saline on Avian Articular Chondrocytes.

The previous result suggested that the high K^+ technique for depolarisation proved effective, and therefore it was then tested to see if the same technique was suitable to study chondrocyte VACC activity. It had been shown that avian growth plate chondrocytes express VACC activity and therefore it was postulated that avian articular chondrocytes may also have detectable VACC activity (Zuscik *et al.*, 1995; Zuscik *et al.*, 1997). Cartilage explants were excised aseptically from lateral condyle and incubated overnight in 1mg/ml collagenase (37°C; 5%:95%; pH 7.4 air: CO_2) releasing the chondrocytes into 380mOsm.kg H_2O^{-1} DMEM (37°C; pH 7.4; see materials and methods). Chondrocytes were then incubated with fura-2 AM (5μM; 37°C; 30mins) and visualised using fluorescence microscopy as previously described.

Cells were perfused for 2mins with the 1mM calcium 'control saline' (380mOsm.kg H_2O^{-1}; to match the extracellular osmolality of cartilage; see section 2.1.0) and a stable baseline recorded. The saline was then changed to the high K^+ saline for 2mins. In response to the depolarisation there was a rise in $[Ca^{2+}]_i$ in some cells where the 358:380nm ratio increased ~40% over basal levels. After a maximal rise, the $[Ca^{2+}]_i$ then began to return to basal levels during the remainder of the experiment period. After 2 mins in the high K^+ saline, the saline was then changed back to the 'control' saline for a period of 2 mins where the cells were allowed to 'recover'. This experimental process was then subsequently repeated and in response to each period in high K^+ saline, the cells responded with a rise in $[Ca^{2+}]_i$ (Fig. 3.11a).

To show that calcium was entering the cell from the extracellular media, the depolarisation experiment was then repeated using a Ca^{2+}-free (2mM EGTA) depolarising saline. As previously described, the chondrocytes were perfused with a 'control' saline for 2mins and a stable baseline recorded. The perfusing saline was then changed to the '1mM calcium high K^+ saline and there was a rise in $[Ca^{2+}]_i$ that then began to return to basal levels during the remainder of the experiment period. The perfusing saline was then changed back to the 'control' saline for a period of 3mins to allow the chondrocytes to 'recover'.

A second high K^+ pulse was then applied using a 2mM EGTA high K^+ saline and this failed to elicit a rise in $[Ca^{2+}]_i$ proving that the $[Ca^{2+}]_i$ rise was due to calcium influx. After a recovery period (3mins) in control saline a third high K^+ pulse was then applied using the original '1mM calcium high potassium' saline and this elicited a rise in $[Ca^{2+}]_i$ (Fig. 3.11b).

These data shown that freshly-isolated avian articular chondrocytes expressed functional VACC and that the rise in $[Ca^{2+}]_i$ was solely due to an influx of calcium from the extracellular environment. These data also confirm that the technique of a high K^+ saline is suitable for the study of VACC in chondrocytes and that therefore these are absent in bovine chondrocytes.

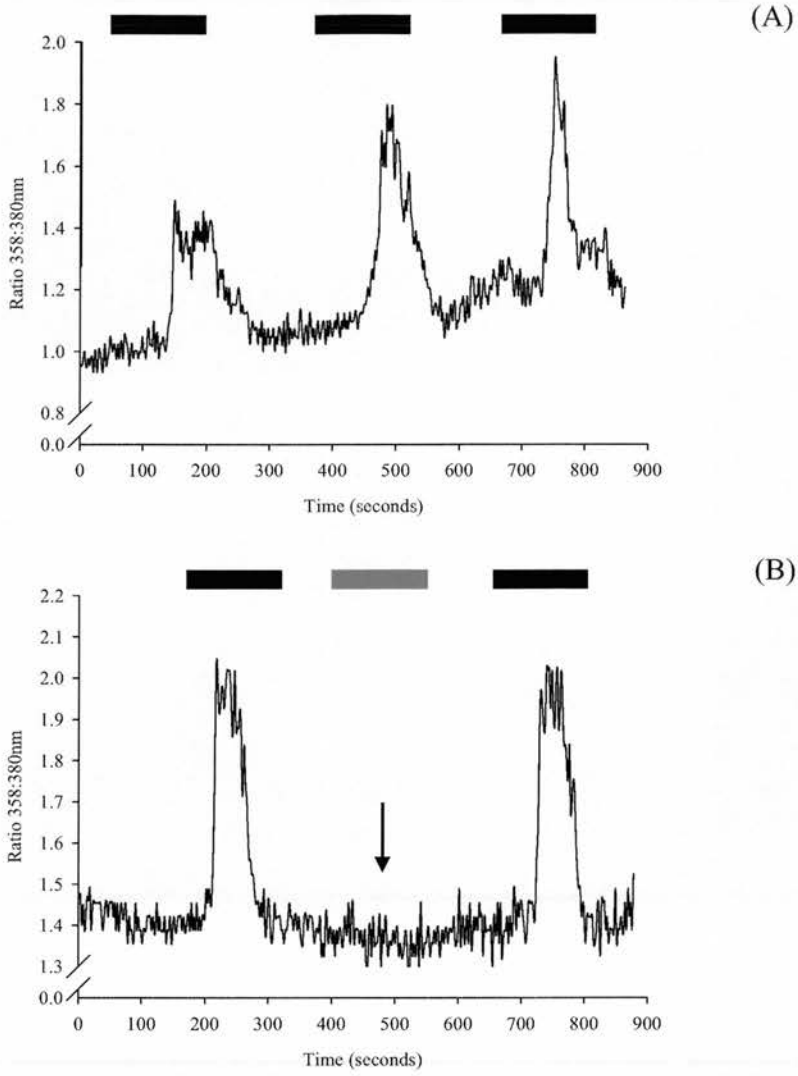


Figure 3.11. Changes in $[Ca^{2+}]_i$ in AACs in response to a high K^+ saline.

ACCs were incubated with fura-2 AM (5μM; 37°C; 30mins) and visualised using fluorescent microscopy (see Materials and Methods). Cells were perfused with a control saline for 2mins and then the perfusion switched to deliver a high K^+ saline. In response to the high K^+ there was a rise in $[Ca^{2+}]_i$ (as shown by the 358:380nm ratio) that subsequently began to return to basal levels (A) There was a slight drift in the baseline and this is most likely the result of the $[Ca^{2+}]_i$ not being allowed to return to 'resting' levels before the next experimental challenge was applied. Using a calcium free (2mM EGTA) high K^+ saline the rise in $[Ca^{2+}]_i$ was abolished indicating the$[Ca^{2+}]_i$ rise was due to calcium influx (B). Black bar = 1mM calcium, high K^+ saline. Red bar = 2mM EGTA high K^+ saline. Data are expressed as raw fluorescent ratio traces and the arrow indicates the lack of a $[Ca^{2+}]_i$ rise in the 2mM EGTA high K^+ saline. N= 10 cells.

3.2.11 A Rise in $[Ca^{2+}]_i$ did not Induce Cell Shrinkage.

The data presented so far would seem to indicate that a rise in $[Ca^{2+}]_i$ is not necessarily a mediator in chondrocyte RVD. It was subsequently tested to determine if the addition of a pharmacological agent known to initiate a rise in $[Ca^{2+}]_i$ in chondrocytes would cause cell shrinkage due to the activation of the RVD response. Chondrocytes were isolated from bovine articular cartilage and incubated with fura 2 AM (5μM; 30mins; 37°C; see Materials and Methods). A steady recording of cell volume (as shown by the 358nm emission) was obtained during perfusing with a 380mOsm.kg H_2O^{-1} control saline. The saline was then switched to a saline containing histamine (100μM; 380mOsm.kg H_2O^{-1}) an agent previously shown to cause a rapid rise in $[Ca^{2+}]_i$ (Higgins *et al.*, 1995; Horwitz *et al.*, 1995; Horwitz *et al.*, 1996). A transient rise in $[Ca^{2+}]_i$ was observed (in some cells) which then returned to basal levels after ~2mins, however there was no change in cell volume as indicated by a stable 358nm intensity throughout the experiment (Fig. 3.12).

These preliminary data would suggest that a rise in $[Ca^{2+}]_i$ is not necessarily a mediator in the activation of RVD response. It is important to note that even though the rise in $[Ca^{2+}]_i$ was similar to that observed in response to a hypo-osmotic stimulus, the experimental situations are not identical. For example, cell swelling results in the unfolding of the plasma membrane as well as membrane stretch, and it is possible that these stimuli are also required to activate the RVD response (Guilak *et al.*, 1999a; Guilak *et al.*, 1999b; Guilak, 2000; Guilak *et al.*, 2002).

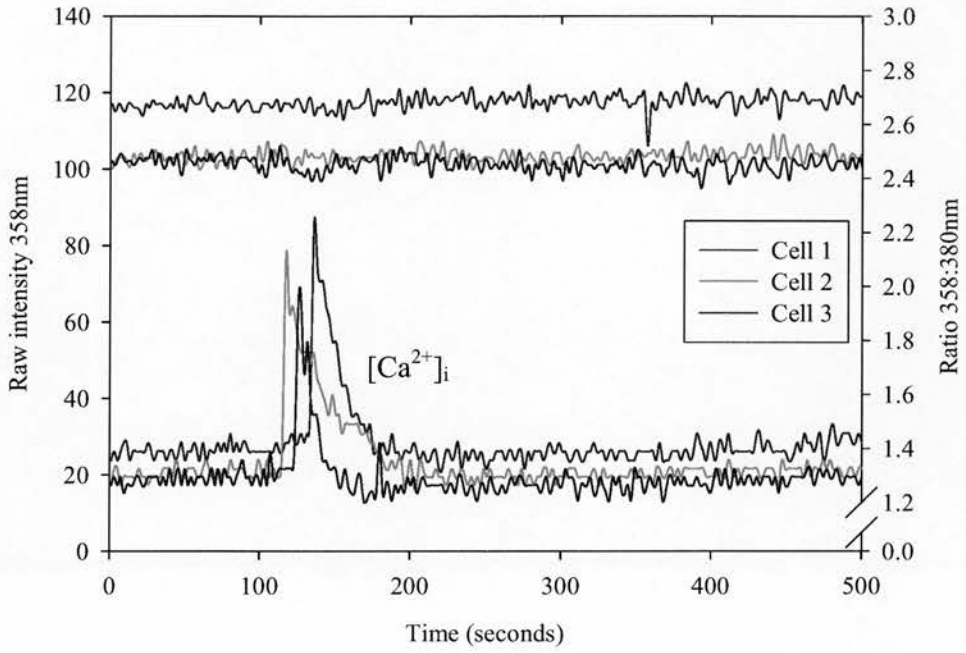


Figure 3.12. Histamine induced $[Ca^{2+}]_i$ rise in chondrocytes.

Chondrocytes were isolated from bovine articular cartilage and incubated with fura 2 (5μM; 30mins). A 'resting' fluorescence was recorded for 2mins under constant perfusion of a control 380mOsm.kg H_2O^{-1} saline and then the saline switched to deliver a 100μM Histamine. The histamine initiated a rise in $[Ca^{2+}]_i$ in some cells that subsequent returned to basal levels. The 358nm fluorescence remained steady therefore suggesting that the $[Ca^{2+}]_i$ transient does not activate the RVD response that would otherwise result in cell shrinkage. Data shown are raw fluorescence traces from 3 cells (2 joints) as an example of the response.

3.3.0 Results Summary.

The data presented in this chapter have shown that both freshly-isolated and 2D cultured chondrocytes have the capacity for RVD in response to a decrease in extracellular osmolality. RVD did not appear to be mediated by stretch-sensitive ion channels as gadolinium had no effect on the number of cells responding, or on the rate of volume recovery. The data presented in this chapter does not rule out the possibility of Gd^{3+} insensitive stretch-sensitive channels and this required further study. Conversely, REV 5901 was a potent inhibitor of the response completely inhibiting RVD in freshly-isolated chondrocytes and significantly decreasing the percentage of cultured cells responding. Removal of extracellular calcium (plus the addition of EGTA) significantly decreased the percentage of cells able to volume regulate although the reason is still unclear.

The decrease in extracellular osmolality initiated a rise in $[Ca^{2+}]_i$ that was not mediated through the activation of VACC or stretch sensitive ion channels, although was due to an influx from the extracellular environment. The percentage of cells showing a $[Ca^{2+}]_i$ rise was not significantly attenuated by a pre-incubation with dantrolene and this had little effect on the number of cells able to volume regulate. The removal of extracellular calcium (plus the addition of 2mM EGTA) significantly decreased the number of cells showing a $[Ca^{2+}]_i$ rise although the response was not completely ablated in all cells. This would seem to indicate that there was a calcium influx from the extracellular environment (as confirmed by the Mn^{2+} technique) and a release of calcium from dantrolene-insensitive intracellular stores.

There did not appear to be a correlation between the rise in $[Ca^{2+}]_i$ and the RVD response as some chondrocytes still underwent RVD without a $[Ca^{2+}]_i$ rise whereas other cells underwent a rise in $[Ca^{2+}]_i$ without any volume regulation. Interestingly, during time in 2D culture, there was a switch in the sensitivity of the $[Ca^{2+}]_i$ response with the rise becoming sensitive to gadolinium and therefore potentially mediated by stretch-sensitive channels.

In summary, the capacity for RVD of both freshly-isolatedand 2D cultured chondrocytes is similar, and there did not appear to be a dependence upon a rise in $[Ca^{2+}]_i$ in most cells. During 2D culture there is was change in the $[Ca^{2+}]_i$ response, moving away from a gadolinium-insensitive activation to one that is sensitive to gadolinium and possibily stretch.

3.4.0 Chapter Discussion.

The data presented in this chapter have shown that freshly-isolated bovine articular chondrocytes have the capacity for RVD following a hypo-osmotic challenge due to a decrease in medium osmolality. The chondrocyte response was varied, with some cells showing RVD and changes in $[Ca^{2+}]_i$ and others not. Furthermore, a key finding from this study was that chondrocytes cultured for up to 3 weeks appear to differ in part of their response to a change in extracellular osmolality due to the development of a Gd^{3+}-sensitive $[Ca^{2+}]_i$ rise and therefore may not be a good model for the study of volume regulation. The implications of chondrocyte culture, the use of freshly-isolated cells and RVD will now be discussed.

Before commenting on the RVD data presented in this chapter, it is important to place freshly-isolated chondrocytes into an *in situ* context. There has been a significant amount of work on the swelling properties and volume-regulatory capacity of *in situ* and isolated bovine articular chondrocytes (Bush & Hall, 2000, 2001b, a; Hall & Bush, 2001). Volume measurements on *in situ* bovine articular chondrocytes have shown that the chondrocyte resting volume increases with the depth of cartilage and that chondrocytes from the SZ swell more than those from the MZ and DZ for a given osmotic challenge (Bush & Hall, 2001a). However, upon release from the extracellular matrix, resting volume stabilises at ~640μm^3 which was significantly larger than the *in situ* volumes for SZ and MZ (resting volume ~454μm^3 and ~550μm^3 respectively) but not the DZ (Bush & Hall, 2001b). This suggests that some chondrocytes within an isolated population would have had to volume-regulate more (following isolation) than others depending upon the zone from which they originated (as cartilage osmolality

follows FCD that increases with cartilage depth (Maroudas, 1980). Therefore, despite no significant difference in resting volume, there will most likely be a difference in the intracellular milieu (due to the efflux of K^+ and Cl^-) as a result of the RVD response. Furthermore, all RVD experiments on isolated chondrocytes will effectively be a second osmotic challenge, and therefore depending on the amount of swelling (or shrinkage) and volume recovery as a result of a release from the matrix may influence the RVD studied in isolated chondrocytes. These points are important when considering the RVD data presented here.

Isolated chondrocyte populations are obviously heterogeneous. They will comprise of chondrocytes from all zones of cartilage but may be biased towards cells from the MZ as these represent ~51% of the chondrocyte population (Hall, 1998). It is therefore tempting to assume that all the cells will behave as MZ cells (i.e show a perfect 'free swelling' Boyle-van't Hoff linear relationship) but this may not be the case. It is not currently known how chondrocytes from the SZ and DZ swell, and it is possible that they do not show this relationship thus making the analysis of an isolated population more complex. The swelling behaviour of chondrocytes from all zones of cartilage therefore needs to be studied. Using *in situ* confocal microscopy it would be possible to study the extent of swelling, RVD and if chondrocytes from all zones show a Boyle-van't Hoff linear relationship.

Having discussed the potential differences of the chondrocytes within an isolated population there is one other important consideration that may have a bearing on this study. As mentioned, MZ chondrocytes will most likely dominate the isolated

chondrocytes population. It is therefore conceivable that should there be any differences in the cellular response between each individual zone, they may be masked as the SZ chondrocytes will only make up ~17% of the population and DZ chondrocytes ~30% (Hall, 1998). Every care has been taken to avoid this as indicated by the heterogeneity of the data presented in this chapter. Therefore, despite using a chondrocyte population, it might be possible to identify individual sub-populations should they exist. The data presented in this chapter suggests there are at least two individual sub-populations with respect to RVD and this point will be discussed later.

As previously mentioned, freshly-isolated bovine articular chondrocytes have a significantly larger cell volume than those *in-situ* but they still show REV 5901-sensitive RVD (Bush & Hall, 2000, 2001b). However, it is possible that the mechanism for RVD between freshly-isolated and *in situ* chondrocytes is different (due to the potential interaction with the ECM), although as the pharmacology and the rate of the response appears the same, this would suggest that the freshly-isolated data shown here would reflect the native cell state (Bush & Hall, 2001b). In contrast to this, chondrocytes cultured for up to 3 weeks would appear to differ in part of their response (due to the development of a Gd^{3+}-dependent $[Ca^{2+}]_i$ rise although no inhibition of RVD; Fig. 3.2.9), and therefore may not be a good model for the study of chondrocyte volume regulation.

Under control conditions (1mM $[Ca^{2+}]_o$) and after a 43% hypo-osmotic challenge by perfusion ~60% chondrocytes were able to regulate their volume and in most cases resulted in a >65% volume recovery in 10 mins; termed 'robust RVD'. Chondrocyte

volume regulation was mediated by what has been termed the 'osmolyte channel', although little is known about the signal transduction pathway(s) that activate or switch off the RVD response or if it is actually a channel (Hall, 1995; Hall *et al.*, 1996a; Hall *et al.*, 1996b; Bush & Hall, 2000, 2001a, 2001b). What is known is that volume regulation can be blocked using the non-specific inhibitor (REV 5901) but again the mechanism of this inhibition is poorly understood (Hall & Kerr, 2000; Kerrigan & Hall, 2000). The data presented here examined the possibility that calcium influences bovine articular chondrocyte RVD.

A hypo-osmotic challenge resulted in a rise in $[Ca^{2+}]_i$ that did not always appear to correlate with the onset of RVD (Fig. 3.5) and in some cases, RVD had already began before the $[Ca^{2+}]_i$ rise reached its maximum. When correlating the changes in $[Ca^{2+}]_i$ to RVD there are four possible outcomes and these are shown in Table 3.4. From the table it is possible to see that the calcium response was heterogeneous and that there are potentially two sub-populations with different dependences on $[Ca^{2+}]_i$ to mediate RVD. It could be argued that the maximal rise in intracellular calcium was not required to activate the 'osmolyte channel' and that the extent of the intracellular calcium rise is secondary to it actually occurring. For example, it could be possible that the maximal rise in intracellular calcium were 15% above 'resting' levels, but a rise of only 5% is sufficient to initiate the RVD response. If this were true, then the initiation of the $[Ca^{2+}]_i$ rise supersedes RVD in some cells and therefore based on these data alone calcium cannot be ruled out as a signalling ion in volume regulation.

RVD	Change in $[Ca^{2+}]_i$	% Chondrocytes showing RVD
Yes	Yes	41
Yes	No	25
No	Yes	26
No	No	8

Table 3. 4. Possible relationships between RVD and change in $[Ca^{2+}]_i$.

When correlating RVD data to changes in $[Ca^{2+}]_i$, there are four possible outcomes. The data from this experimental chapter is also included showing the percentage of chondrocytes exhibiting RVD correlated to changes in $[Ca^{2+}]_i$, recorded in a 1mM 'control saline'. N=107 cells.

One would assume that cells not showing RVD would be deficient in either the signal transduction pathway required to detect a hypo-osmotic change, initiate a response or it is possible that the cells are just dying.. Cell preparations in this study were routinely checked for cell viability (prior to experimentation) using trypan-blue staining where it is found that viability often exceeded 95% (data not shown). In addition, as a cell dies, the plasma membrane would eventually become leaky and so the cells would not retain a fluorescent probe and thus under imaging the intensity signal would fall (due to dye

leakage). Under the described criteria (see Materials and Methods), the cell would not have been used for analysis and consequently the sample remained unbiased. The fact that there was no significant difference in the calcium response for 'responding' or 'non-responding' chondrocytes in response to a hypo-osmotic challenge (Fig. 3.2.8) would support the theory that a calcium rise was not a mediator of RVD. There are some examples in the literature where RVD proceeds independently of calcium where experiments were performed by removing extracellular calcium and adding either EGTA of BAPTA. For example, rat cerebellar granule neurones exhibit complete RVD independent of extracellular calcium and the hypo-osmotically induced transient $[Ca^{2+}]_i$ rise (Moran *et al.*, 1997; Morales-Mulia *et al.*, 1998). RVD was measured by coulter counter and ^{125}I efflux, and was shown to be insensitive to Ca^{2+} blockers (including Gd^{3+}, Verapmil, La^{3+}) the removal of extracellular calcium or by the addition of BAPTA-AM. It was therefore concluded that the volume regulatory response was not mediated by changes in intracellular calcium in this cell-type.

If the calcium rise were involved in the RVD response, the inhibition of the rise should attenuate RVD. No added calcium plus the addition of 2mM EGTA significantly inhibited the number of cells showing a rise in $[Ca^{2+}]_i$. Furthermore, as the Mn^{2+} experiments showed quenching in response to a decrease in extracellular osmolality this provides a strong case for calcium influx. This observation is in agreement with other studies on the mechano-induced $[Ca^{2+}]_i$ transients in response to changes in osmolality, fluid flow and direct mechanical deformation in chondrocytes (Yellowley *et al.*, 1997; Guilak *et al.*, 1999b; Yellowley *et al.*, 1999; Yellowley *et al.*, 2002). Interestingly, in the 2mM EGTA saline, some cells (albeit only a few) still exhibited a rise in $[Ca^{2+}]_i$.

The addition of dantrolene (inhibitor of ER calcium release) did not prevent the rise and therefore and the $[Ca^{2+}]_i$ transient was most likely the result of a calcium release from a dantrolene-insensitive intracellular store. One possible pathway has been shown in porcine articular chondrocytes, where a decrease in extracellular osmolality initiated a x5 fold increase in intracellular IP_3 (Erickson *et al.*, 2003). Therefore in bovine articular chondrocytes, the rise in $[Ca^{2+}]_i$ may be attributed to this pathway (Erickson *et al.*, 2001). Having established a potential mechanism for the $[Ca^{2+}]_i$ rise, the percentage of chondrocytes showing RVD was then investigated in the 2mM EGTA, calcium-free saline.

The percentage of chondrocytes responding with RVD in the hypo-osmotic 2mM EGTA saline was significantly less than in the control 1mM Ca^{2+} saline (Table 3.1). If calcium had no role in the capacity for RVD then one would expect the number of responding cells to be the same. The fact that there was a decrease in the percentage of cells responding may imply that for some chondrocytes, the rise in calcium is required to mediate RVD. Interestingly, upon the osmotic challenge there was a decrease in the 358:380nm ratio in some cells that was most likely the result of the chelation of $[Ca^{2+}]_i$ (average standardised decrease of -3.4 ± 0.31%). This lowering of intracellular calcium may attenuate the cellular response, as calcium is critical to metabolism and cellular organisation. For example, $[Ca^{2+}]_i$ is crucial in the regulation of the cell actin cytoskeleton, and as the actin cytoskeleton has been shown to be involved chondrocyte mechanotransduction (Wright *et al.*, 1996; Millward-Sadler *et al.*, 1999), a decrease in $[Ca^{2+}]_i$ may inhibit these responses.

It is conceivable that as cartilage is not a homogeneous tissue (i.e. varies in structure and composition with depth) the chondrocyte response to changes in osmolality differs between the three main zones. The freshly-isolated RVD data suggests the existence of at least two sub-populations, one dependent upon a rise in $[Ca^{2+}]_i$ (i.e. calcium dependent RVD response), and the other independent (i.e. calcium independent RVD). In an EGTA saline, 22.96 ± 5.11% of cells showed RVD (Table 3.1). Data from the 1mM calcium experiments have shown that of the ~60% of chondrocytes that preformed RVD, ~41% underwent RVD with a rise in $[Ca^{2+}]_i$ and ~25% showed RVD independent of a rise in $[Ca^{2+}]_i$ (data are for whole cell populations therefore not possible to calculate and error; n=107 cells). When comparing these values, there appears to be a correlation with the percentage of cells showing RVD with out a rise in $[Ca^{2+}]_i$ and those that volume regulated in the EGTA saline. This in conjunction with the fact that there were no significant differences between the change in $[Ca^{2+}]$ between cells that did and did not shown RVD, may therefore suggest two sub populations of chondrocytes with difference dependences on $[Ca^{2+}]_i$ for RVD.

In addition, the Mn^{2+} quench data further highlighted the differences in dependence on $[Ca^{2+}]_i$ for the RVD response. Quenching was observed in response to the hypo-osmotic challenge (as seen by the fall in the 358nm emission) suggesting an opening of a plasma membrane calcium channel. In some cells there was no quenching, and in fact it was possible to observe RVD. As the 358nm emission decreases should Mn^{2+} enter the cell, one can assume that these cells (~14% of chondrocytes) are not responding with a rise in $[Ca^{2+}]_i$ but were showing RVD. This was therefore a calcium-independent RVD response.

Slightly conflicting data was obtained from the histamine experiments (Fig. 3.12). A rise in $[Ca^{2+}]_i$ did not result in cell shrinkage due to the activation of the 'osmolyte channel'. There are two possible explanations for this. By chance, the cells selected did not exhibit calcium-dependent RVD, and therefore repeating the experiment with an increased number of cells may resolve this. Or secondly, it is conceivable that a change in $[Ca^{2+}]_i$ is only part of the signal pathway required to mediate RVD and therefore by raising $[Ca^{2+}]_i$ alone the RVD response would not be activated. In intestinal 104 cells it has been commented that histamine stimulation of $p125^{FAK}$ was not enough to stimulate the RVD response and therefore it may also require a second signal (Tilly *et al.*, 1996).

Having showed RVD in freshly-isolated chondrocytes, the effect of 2D culture was subsequently investigated. Chondrocytes that had been isolated and cultured in monolayers for 3 weeks retained the capacity for RVD. Furthermore, the response was inhibited by REV 5901 and this strongly suggests the same pathway was used. Interestingly, time in culture did influence the hypo-osmotic induced $[Ca^{2+}]_i$ transient. In freshly-isolated chondrocytes, the rise in $[Ca^{2+}]_i$ was not inhibited by Gd^{3+} suggesting it was not mediated by a stretch-sensitive channel. However, in 2D cultured chondrocytes, Gd^{3+} inhibited the rise. This is a significant finding for two reasons. The first is that time in culture changes the chondrocyte calcium response and therefore these cells may not behave as *in vitro* chondrocytes. This may suggest that experiments on cultured chondrocytes may be misleading when relating the data back to the chondrocytic phenotype. The second important finding was that even though the rise in $[Ca^{2+}]_i$ was inhibited, RVD was not. This separation of the RVD response from the rise

in $[Ca^{2+}]_i$ may imply that in cultured chondrocytes calcium is not involved in RVD. This then raises the question as to why does intracellular calcium change. One possibility would be that the calcium rise is involved in the regulation of the F-action cytoskeleton (as shown by Erickson *et al.*, 2003) although at this stage, this is pure conjecture.

Due to experimental limitations, it was not possible to record changes in $[Ca^{2+}]_i$ and volume simultaneously in 2D chondrocytes as the fura-2 fluorescence was not great enough (unpublished observations). With this in mind, it is not possible to state with complete confidence whether RVD in 2D cultured chondrocytes was calcium-independent for all cells. One could speculate that time in culture results in the de-differentiation of the chondrocyte population from one consisting of two sub-populations to one that exhibits a more uniform response. Furthermore, it could be suggested that there was a switch from a stretch insensitive phenotype to one where stretch is involved in mechanotransduction. In cultured human articular chondrocytes, pressure induced strain activates a Gd^{3+} sensitive, integrin regulated pathway (Wright *et al.*, 1996; Millward-Sadler *et al.*, 1998b). In the present study, as Gd^{3+} did not influence the rise in $[Ca^{2+}]_i$ in freshly-isolated chondrocytes this pathway is most likely not present. Conversely, in 2D cultured chondrocytes the response may be similar to that of human chondrocytes. Solely, based on the data present in this chapter, it is not possible to determine if this is true and further work needs to be undertaken.

Recently a study on 2D cultured BACs has shown that Gd^{3+} inhibited the rise in $[Ca^{2+}]_i$ as well as attenuating the capacity for RVD (Yellowley *et al.*, 2001, 2002). Here, it has been shown that the rise $[Ca^{2+}]_i$ was inhibited, but the capacity for RVD was

maintained. As these results appear to conflict the data presented here, this will be addressed. The discrepancy could be due to a difference in culture conditions. In this study, only 10% FCS was used compared to 20% by Yellowley *et al* (2001). As FCS has been shown to influence the properties of chondrocytes; (Vivien *et al.*, 1991), the presence in the culture media may influence the cell response to culture and subsequently changes in osmolality. It is also possible that differences in experimental protocol could be involved. Here, RVD in 2D cultured cells was studied after ~12 hours post-passage at 37°C whereas in the aforementioned study, RVD was studied in freshly passaged cells at RT°C. It is possible this will make a difference in the response although currently it is not clear how. Another possibility would be the difference in the osmotic challenge. In this study a 43% hypo-osmotic challenge was applied compared to 50%. It is possible that at the lower osmotic challenge the membrane was not stretched enough (as it resides in folds; Guilak *et al.*, 2002) and therefore the RVD response was not inhibited by Gd^{3+}. Increasing the osmotic challenge may therefore result in the stretching of the membrane and the subsequent inhibitory effects of Gd^{3+}. All of these possibilities need to be examined to further understand the difference in the results.

In summary, it has been shown that both freshly-isolated and 2D cultured chondrocytes have the capacity for RVD and that time in culture results in a change in the sensitively of the calcium response to the SAC inhibitor Gd^{3+}. The decrease in osmolality results in a calcium influx from the extracellular environment mediated by a non-voltage dependent calcium channel. Furthermore, the decrease in extracellular osmolality

initiates a release of calcium from dantrolene-insensitive calcium stores. The rise in $[Ca^{2+}]_i$ did not appear to correlate with the capacity for RVD and may either be an artefact or two sub populations of chondrocytes were RVD is dependent on calcium in one and not for the other.

The mechanism of chondrocyte RVD is still to be elucidated. The maintenance of cell volume (and the intracellular milieu) is crucial to optimal metabolism and deviations from this may exacerbate the degeneration process. Further work is required to understand the exact mechanism of the REV 5901 inhibition of the RVD response and to understand the possible link between RVD and the chondrocytic phenotype. *In situ* confocal studies examining changes in $[Ca^{2+}]_i$ in relation to cartilage zone would further the understanding as why there appears to be at least two separate sub-populations with respect to RVD. Understanding the signalling processed involved would help in the comprehension of chondrocyte mechanotransduction and how the cells perceive and respond to the physico-chemical environment.

Regulatory Volume Increase (RVI)

4.1.0 Chapter Introduction

The capacity for a cell to volume regulate towards its 'set-point' in response to an increase in extracellular osmolality is termed ***Regulatory Volume Increase*** (RVI; (O'Neill, 1999). Various membrane transporters have been implicated in the RVI response (see section 1.7.0) including the Na^+-K^+-$2Cl^-$ cotransporter (NKCC; isoform $NKCC_1$) and the Na^+-H^+ exchange (principally NHE1). In chondrocytes, immunohistochemistry has shown that both of these transporters are expressed (Dascalu *et al.*, 1993; Dascalu *et al.*, 1996; Trujillo *et al.*, 1999), whose activity can be stimulated by changes in extracellular osmolality (Errington & Hall, 1995; Wilkins *et al.*, 1995b; Hall *et al.*, 1996b; Wilkins *et al.*, 1996; Errington *et al.*, 1997; Browning *et al.*, 1999). Upon cell shrinkage, these transporters mediate the influx of ions and consequently osmotically obliged water from the extracellular environment into the cell. This subsequently results in cell swelling and the restoration of cell volume. In chondrocytes, a direct link between the capacity for RVI and the expression of these transporters is yet to be established and therefore this will be the focus of this piece of work.

An increase in extracellular osmolality results in a decrease in matrix synthesis when measured by ^{3}H-proline and ^{35}S-sulphate incorporation in isolated chondrocytes (Urban *et al.*, 1993). The reason for the decline is currently unknown, although after a period of 2-16 hours, there is a recovery in synthesis where the new optimal rate is at the isolation osmolality (Urban *et al.*, 1993). This would seem to suggest that chondrocytes have the capacity to adapt to changes in extracellular osmolality and maintain the synthesis of the extracellular matrix. Optimal cell metabolism is closely related to the intracellular ionic environment, and therefore deviations (either an increase or decrease in ion

concentration) will have a detrimental effect on metabolism. It has been suggested by Urban *et al.,* (1993) and Hall & Bush (2001) that there may be a link to intracellular K^+ and matrix synthesis based on observations made by Horowitz & Lau (1988) although this is still to be proven (Horowitz & Lau, 1988; Urban *et al.*, 1993; Hall & Bush, 2001). Therefore, a possible mechanism that may account for the recovery in rate of synthesis would be the restoration of cell volume via RVI.

Currently, in bovine articular chondrocytes, it is unknown if the cells have the capacity for RVI, and if so the signalling pathways are still to be identified. It has been shown that chondrocytes respond to mechanical stimulation with a rise in $[Ca^{2+}]_i$ (Yellowley *et al.*, 1997; Guilak et al., 1998; Guilak *et al.*, 1999b; Yellowley *et al.*, 1999; Edlich *et al.*, 2001) and in chondrocytes it has been shown that a increase in osmolality initiates a transient rise in $[Ca^{2+}]_i$ (Erickson *et al.*, 2001). Interestingly, inhibition of the $[Ca^{2+}]_i$ by the removal of extracellular calcium has been shown to further stimulate volume recovery in response to a hyper-osmotic stimulus although it is not clear if this was by direct effect (Erickson *et al.*, 2001). It is therefore possible that changes in $[Ca^{2+}]_i$ are involved in mediating the RVI response forming part of the signalling transduction pathway.

In this chapter, the capacity for RVI was studied in freshly-isolated and 2D cultured chondrocytes in response to an increase in extracellular osmolality (380mOsm.kg H_2O^{-1} to 540mOsm.kg H_2O^{-1}) by NaCl addition. Using fura-2, both changes in cell volume and $[Ca^{2+}]_i$ were recorded and the capacity for RVI correlated to any changes in $[Ca^{2+}]_i$. The expression of the NKCC was investigated by SDS-gel electrophoresis using a

specific antibody to the NKCC, and the role of the NKCC cotransporter in RVI was studied using 75μM bumetanide (a specific inhibitor).

Hypothesis to be tested:

Chondrocytes have the capacity for RVI and it is mediated by the NKCC cotransporter.

4.2.0 Results

4.2.1 Images of Isolated Fura-2-Loaded Chondrocytes.

Chondrocytes were isolated from full-depth bovine articular cartilage explants (as previously described; see Materials and Methods) and incubated with fura-2 AM (5μM; 30mins; 37°C). Cells were then transferred to a heated stage (37°C) of a Nikon microscope and images acquired using a PTI ***Imagemaster™*** system through an x40 oil objective focused through the midplane of the cells. The incorporated fura-2 was excited through a 400nm dichroic filter at alternating wavelengths of 358nm (Isobestic point $[Ca^{2+}]_i$ insensitive; volume-sensitive wavelength) and 380nm (Volume-sensitive; $[Ca^{2+}]_i$ sensitive; volume sensitive wavelength) at 0.4Hz.

When visualised, the chondrocytes appeared spheroidal with an apparent heterogeneity in cell size (Fig. 4.1). Throughout the main body of the cell, the fura-2 appeared evenly distributed with no areas of intense fluorescence. The fact the dye appeared homogeneous and in conjunction with the linear relationship between fluorescence and osmolality (section 2.3.6) would imply that fura-2 (and the method of dye loading) was suitable for the measurement of cell volume.

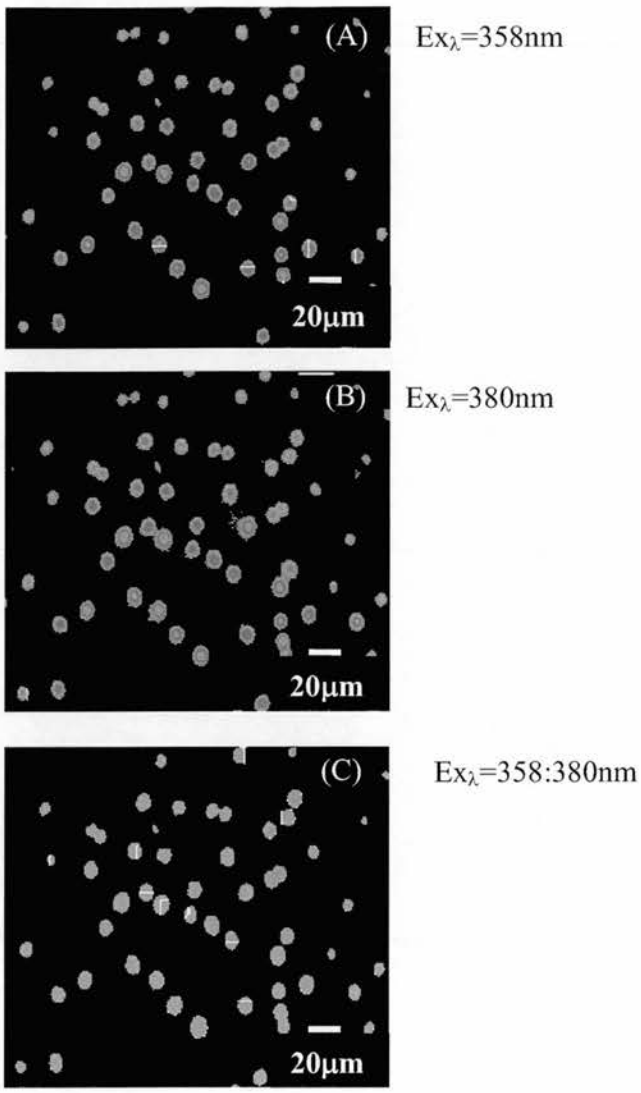


Figure 4.1. Fluorescent images of Fura-2 loaded chondrocytes.

Chondrocytes *were isolated from bovine articular cartilage, incubated with fura-2 AM (5μM; 37°C, 30mins) and visualised by fluorescent microscopy. The fura-2 was excited alternately through a 400nm dichroic filter at (A) 358nm, (B) 380nm and the ratio 358:380nm calculated by PTI software (C). When visualised through a single z-section (reference to the x-y-z planes where x & y are positions in 2D and depth is denoted by the z-plane) the cells appeared rounded with some heterogeneity in apparent cell diameter. The fura-2 was evenly distributed with no areas of high fluorescent intensity that would otherwise indicate compartmentalisation. Images are shown as raw fluorescence on a fluorescent pixel intensity scale of 0-255Au. Cells were from one joint isolated from full depth explants.*

4.2.2 Example of a Chondrocyte Performing Hyper-osmotic RVI.

Chondrocytes were isolated from full depth, bovine articular cartilage explants and incubated with fura-2 AM (5μM; 30mins; 37°C; see Materials and Methods). The coverslips (contained with steel rings) were then transferred to the stage of a Nikon microscope and visualised by fluorescent microscopy. The resting fluorescence (V_{rest}) was recorded under constant perfusion with a 380mOsm.kg H_2O^{-1} 1mM calcium 'control saline' (37°C;) for 2mins and then the saline switched to deliver a hyper-osmotic challenge (380mOsm.kg H_2O^{-1} to 540mOsm.kg H_2O^{-1}; osmolality was increased by NaCl addition). Data were then recorded for a subsequent 10mins, saved and transferred to Excel™ and MAX™ for analysis (see Materials and Methods).

As shown in Figure 4.2, the change in saline resulted in a rapid increase in 358nm fluorescence (measured at 510nm) indicating a decrease in cell volume. The cell response was then recorded for 10mins following the osmotic challenge. The maximal change in cell volume (V_{max}) occurred approximately 60 seconds after the osmotic challenge with a 15.5 % increase in intracellular fluorescence. The cell then volume-regulated by RVI and at the end of the experimental period the change in fluorescence had recovered by 57.5 % (V_{final}). The example shown in Figure 4.2 is that of a single chondrocyte performing RVI, although in most chondrocytes this response was unusual. Most cells did not have the capacity for RVI and this is shown later in section 4.2.3.1.

In response to the increase in extracellular osmolality there was a small change $[Ca^{2+}]_i$ as shown by the red line in Figure 4.2. This would seem to imply that in this example,

the chondrocyte was able to undergo RVI independent of a rise in $[Ca^{2+}]_i$. It is important to note the heterogeneity of the chondrocyte response to an increase in extracellular osmolality. As described in chapter 3, the chondrocyte population was heterogeneous. There were cells that underwent RVI and others that did not appear to have the capacity for volume-regualtion within the time period studied. Furthermore, there were some cells that responded to an increase in osmolality with a rise in $[Ca^{2+}]_i$ whereas in other cells no change was recorded. These points will be further illustrated in section 4.2.4.

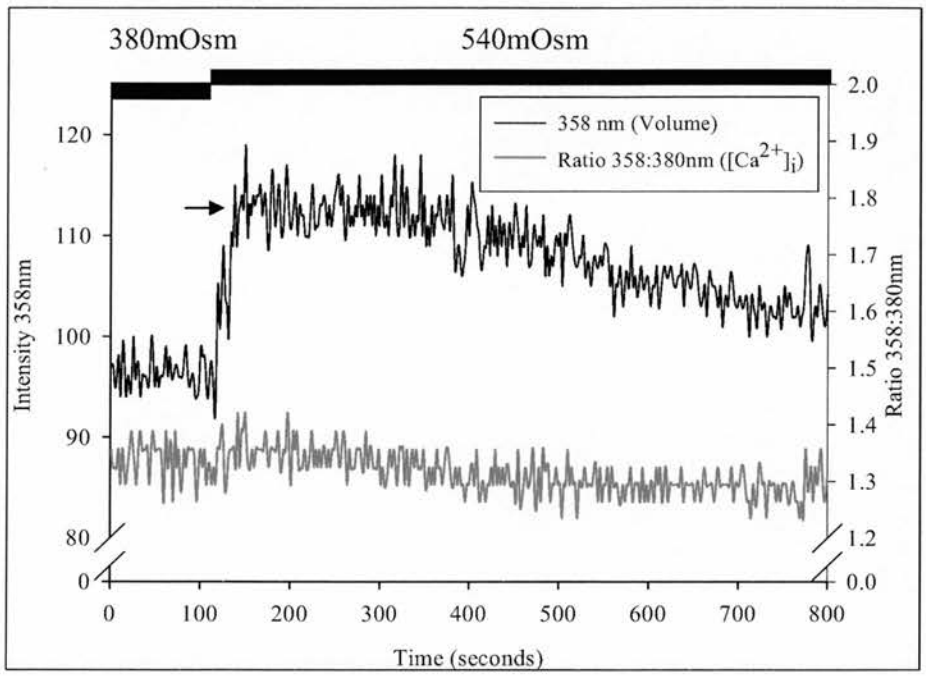


Figure 4.2. Example of a chondrocyte volume regulating by RVI.

Chondrocytes isolated from bovine articular cartilage were incubated with fura-2 AM (5μM; 30mins; 37°C) and visualised by fluorescent microscopy. Cells were perfused with an iso-osmotic saline (380mOsm.kg H_2O^{-1}) for 2 mins and then the saline switched to deliver a hyper-osmotic challenge (380mOsm.kg H_2O^{-1} to 540mOsm.kg H_2O^{-1}). In response to the hyper-osmotic challenge there was an increase in the 358nm emission (as shown by the black line) indicating a decrease in volume. The maximum change in volume (termed V_{max}) occurred ~ 60 seconds after the hyper-osmotic challenge and is shown by the black arrow. The cell then underwent RVI where after 10 mins there had been 57.5 % volume recovery. As shown by the red line, the increase in osmolality did not elicit an $[Ca^{2+}]_i$ transient and therefore in this example it would appear that RVI is independent of a rise in $[Ca^{2+}]_i$. Data are expressed as raw fluorescence. N = 1 cell.

4.2.3 Example of a Chondrocyte performing 'Post RVD-RVI'.

In response to a direct hyper-osmotic challenge, very few cell types are able to perform RVI and the 'post RVD–RVI' protocol is often used to stimulate the response (O'Neill, 1999). The 'post RVD-RV'I protocol is where prior to the application of a hyper-osmotic stimulus a hypo-osmotic challenge is first applied. After the set period in the hypo-osmotic saline, the saline is then returned to the initial iso-osmotic saline that is now effectively hyper-osmotic. Chondrocytes were isolated from bovine articular cartilage and experiments performed as previously described. Data were recorded under constant perfusion with an iso-osmotic 'control saline' (37°C; 380mOsm.kg H_2O^{-1}) for 2mins and then switched to deliver a hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}). After 10mins, the perfusion saline was then switched back to the original saline of 380mOsm.kg H_2O^{-1}.

As shown in Figure 4.3, the decrease in extracellular osmolality resulted in a fall in 358nm fluorescence indicating an increase in cell volume. The maximal change in fluorescence occurred approximately 1.5mins after the osmotic challenge where there was a decrease of 12.82 % (shown as V_{min}). The chondrocyte then underwent RVD where at 10mins post-osmotic challenge volume had recovered by 90.4 %. The saline was then switched to deliver a hyper-osmotic challenge which resulted with an increase in 358nm fluorescence of 11.51 % indicating cell shrinkage (shown as V_{max}). The chondrocyte then underwent RVI and after 10mins, volume had recovered by 61.68 %.

Both the hypo and hyper osmotic challenges resulted in a transient rise in $[Ca^{2+}]_i$ that appeared to correlate with the onset of volume regulation. In this example, when comparing the changes in $[Ca^{2+}]_i$ as a result of a hypo and hyper-osmotic challenges, there were no differences in either the maximal $[Ca^{2+}]_i$ rise or the duration of the response (Fig. 4.3). The rise in $[Ca^{2+}]_i$ as a result of a hypo-osmotic challenge lasted for 164.0 seconds and the maximal point was 22.9 % over basal levels. The second intracellular calcium rise (as a result of the hyper-osmotic challenge) lasted for 165.4 seconds and the maximal rise was 25.6 % over basal levels.

Having shown single cell examples of direct RVI (section 4.2.2) and post RVD-RVI (section 4.2.3), the capacity for chondrocyte RVI was subsequently studied in single cell populations (section 4.3.0). Comparisons are also made between freshly-isolatedand 2D cultured chondrocytes and the expression of the NKCC was studied.

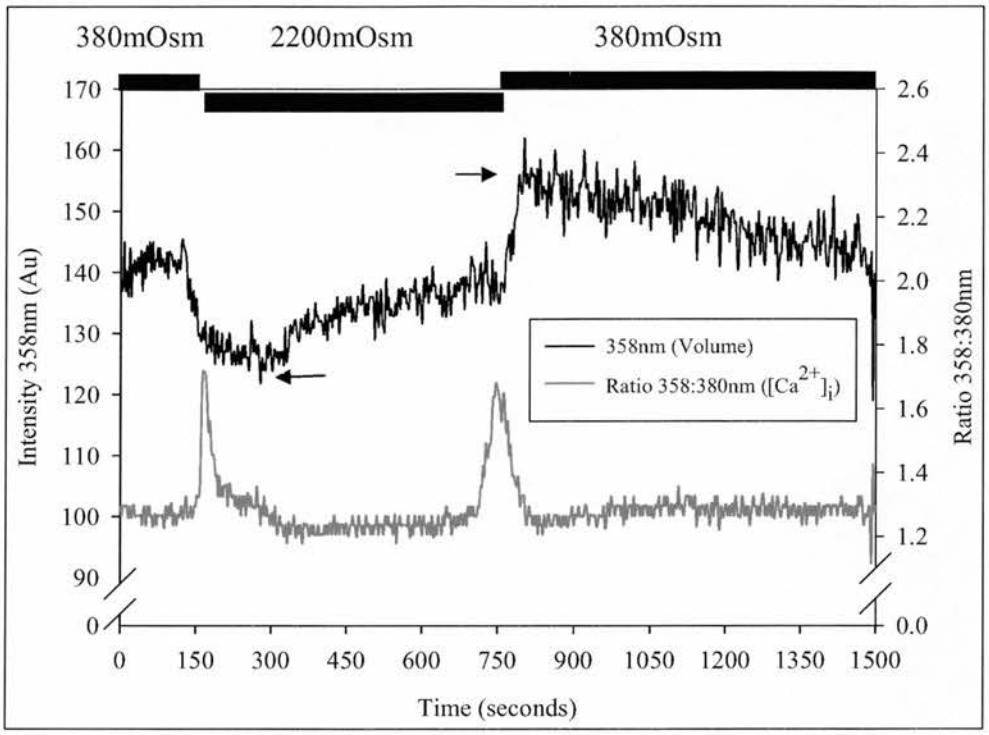


Figure 4.3. Example of a chondrocyte performing Post RVD-RVI.

Chondrocytes were isolated from bovine articular cartilage, incubated with fura-2 AM (5μM; 30mins; 37°C) and RVI studied in response to the 'post RVD-RVI' protocol. Data were recorded for 2mins under iso-osmotic conditions (380mOsm.kg H_2O^{-1}) and then a 43% hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) was applied. This resulted in a decrease in 358nm fluorescence indicating an increase in cell volume (V_{max}). RVD then proceeded where after 10mins volume had recovered by 90.4 %. The saline was then switch to deliver a hyper-osmotic challenge (220mOsm.kg H_2O^{-1} to 380mOsm.kg H_2O^{-1}) and this resulted in an increase in 358nm fluorescence indicating a decrease in cell volume (V_{min}). RVI then proceeded where after 10mins there had been 61.68 % recovery in volume. In both cases, the maximal change in volume is indiciated by a black arrow. The hypo-osmotic challenge resulted in a transient increase in $[Ca^{2+}]_i$ lasting for 164 seconds with a maximal increase of 22.9 % over basal levels. The hyper-osmotic challenge also resulted in a transient rise in $[Ca^{2+}]_i$ that lasted for 165.4 seconds with a maximal rise of 25.6 % over basal levels. Data are expressed as raw fluorescent traces. N=1 cell.

4.2.3 RVI in Isolated Bovine Articular Chondrocytes.

RVI was studied in chondrocytes isolated into 380mOsm.kg H_2O^{-1} DMEM and those isolated into 280mOsm.kg H_2O^{-1} DMEM as it has been suggested that a lower isolation osmolality may stimulate the RVI response (O'Neill, 1999). Cells from bovine articular cartilage were isolated into either 380mOsm.kg H_2O^{-1} or 280mOsm.kg H_2O^{-1} DMEM (as previously described; see Materials and Methods) and RVI recorded in response to a direct hyper-osmotic stimulus and the 'post RVD-RVI' protocol by fluorescent microscopy. Cells were perfused with an iso-osmotic saline for 2mins and then the saline switched to deliver an osmotic challenge. For each osmotic challenge, data were recorded for 10 mins, saved, and subsequently transferred to Excel™ and MAX™ for analysis. A cell was deemed to show RVI if after 10mins there had been at least a 50% recovery in volume from the maximal change.

4.2.3.1 Direct hyper-osmotic challenge

A control saline was perfused for 2mins and then the osmotic challenge applied. For chondrocytes isolated into 380mOsm.kg H_2O^{-1} DMEM, cells were perfused with a saline of 380mOsm.kg H_2O^{-1} for 2mins and then the perfusion saline switched to deliver a saline of 540mOsm.kg H_2O^{-1}. For chondrocytes isolated in 280mOsm.kg H_2O^{-1} DMEM, cells were perfused with a saline of 280mOsm.kg H_2O^{-1} for 2mins and then the perfusion saline switched to deliver a saline of 420mOsm.kg H_2O^{-1}.

In response to the direct hyper-osmotic challenge there was an increase in 358nm fluorescence (as recorded by the 510nm emission) indicating a decrease in cell volume. When comparing the extent of cell shrinkage between chondrocytes isolated into 280mOsm.kg H_2O^{-1} DMEM and those isolated into 380mOsm.kg H_2O^{-1} DMEM, there was no significant difference (Student's t-test; $p>0.05$), with changes of 9.8 ± 1.2 % and 7.5 ± 0.71 % respectively (n=8, N=138). For both experimental groups, some chondrocytes subsequently underwent RVI (Table 4.1).

Data were plotted and the rate of RVI (expressed as a t½) was calculated by linear regression ($r^2>0.9$; time taken for volume to be recovered by 50%; see Materials and Methods). Chondrocytes isolated into 380mOsm.kg H_2O^{-1} DMEM underwent RVI with a t½ of 6.1 ± 0.6 mins (n=5, N=86). This was then compared to chondrocytes isolated into 280mOsm.kg H_2O^{-1} and no significant difference was found (Student's test; $p>0.05$). This would imply that a lower isolation osmolality did not stimulate RVI in isolated chondrocytes.

Despite the fact that there was no significant difference in the rate of RVI between the two experimental groups, using a lower isolation osmolality may have altered the percentage of cells responding. For chondrocytes isolated into 380mOsm.kg H_2O^{-1} DMEM, 6.5 ± 4.15 % of cells underwent RVI (n=5, N=86) compared to 5.26 ± 5.26 % of chondrocytes isolated into 280mOsm.kg H_2O^{-1} media (n=3, N=52). There was no significant difference ($p>0.05$) between these two experimental groups. The large standard errors (particularly in the 280mOsm.kg H_2O^{-1} data) are due to the fact that statistical tests were performed between joints (see Materials and Methods) and in some

experiments no chondrocytes exhibited RVI (Table 4.1). This therefore introduces a large variation in the data set (due to the introduction of zeros) when calculating the percentage responding. This does provide a better estimation of the parent population, whereby using the number of cells would decrease the s.e.m but would be less representative of the actual parent population.

When comparing the extent of RVI over the 10 minute experimental period for each cell (Fig. 4.4), and then between each isolating osmolality, no significant difference (Student's t-test; $p>0.05$) in the distribution was found. In most cells, RVI was completely absent and in many of the cells there appeared to be continued shrinkage (as shown by a rise in 358nm fluorescence) throughout the experimental period (as shown in Fig 4.4; left of the centre red line). For the cells that did show RVI, most cells only recovered their volume by 20-30% with very few cells showing robust RVI. This analysis further illustrates the heterogeneity of the chondrocyte population and may relate to the cartilage zone from which each cell originally resided.

In summary, an increase in extracellular osmolality resulted in a decrease in cell volume as shown by a fall in the 358nm fluorescence. Most cells did not show robust RVI and the response was not further stimulated using a lower isolation osmolality. A few cells did exhibit RVI and some were able to completely volume regulate within the 10min experimental period.

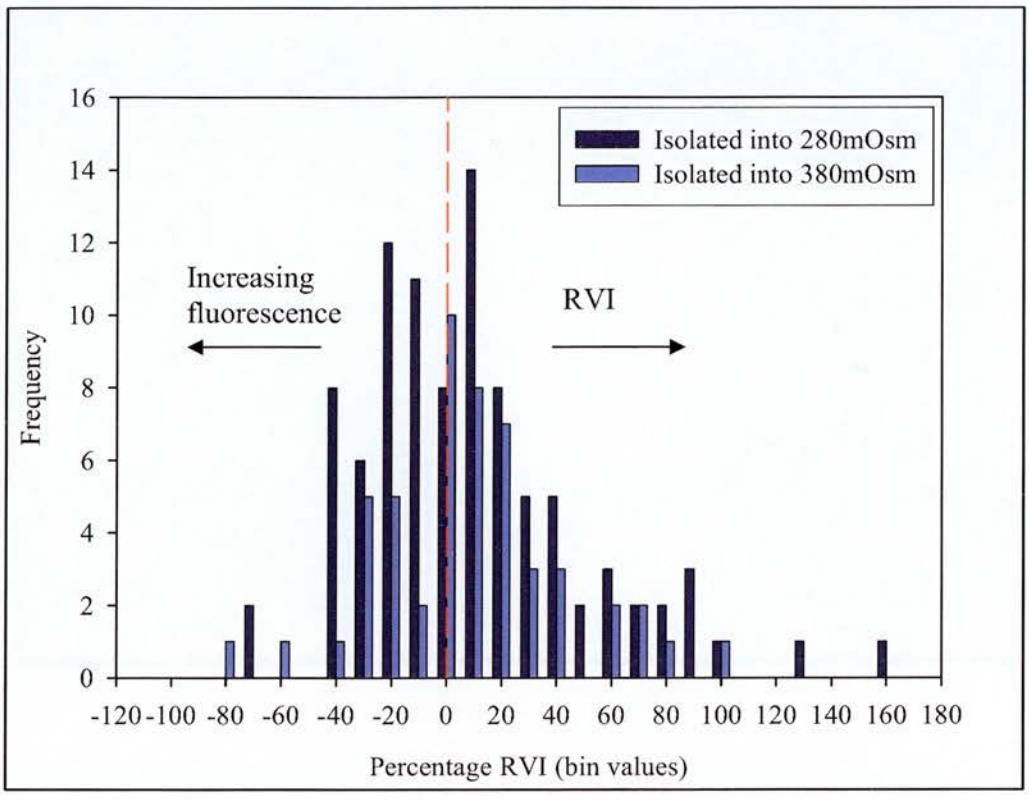


Figure 4.4. Extent of RVI in response in response to a hyper-osmotic challenge.

Bovine articular chondrocytes were isolated into either 380mOsm.kg H_2O^{-1} or 280mOsm.kg H_2O^{-1} DMEM, incubated with fura-2 AM (5μM; 30mins; 37°C) and RVI studied by fluorescent microscopy. In response to the osmotic challenge, there was an increase in 358nm fluorescence indicating a decrease in cell volume. Some cells subsequently exhibited RVI. The extent of volume recovery was heterogeneous, although parametric in distribution for both experimental groups. For chondrocytes isolated into 380mOsm.kg H_2O^{-1} only 6.5 ± 4.2 % exhibited robust RVI and this was not significantly increased ($p>0.05$) by isolating the cells in 280mOsm.kg H_2O^{-1} DMEM where only 5.26 ± 5.26 % responded. Most cells did show signs of slight RVI with volume recovery up to 20-30 % during the experimental period. Data are expressed as means ± s.e.m; n= 8 joints 138 cells.

4.2.3.2 Post RVD-RVI.

As isolating the chondrocytes into a DMEM of a lower osmolality did not stimulate more cells to show RVI, it was subsequently tested to see if more cells responded with RVI to the 'post RVD-RVI' protocol. Furthermore, the effect of isolating into 280mOsm.kg H_2O^{-1} DMEM on the 'post RVD-RVI' was tested. For cells isolated into 280mOsm.kg H_2O^{-1} DMEM, chondrocytes were perfused with a control saline of 280mOsm.kg H_2O^{-1} for 2mins and then the saline switched to deliver a hypo-osmotic stimulus (280mOsm.kg H_2O^{-1} to 140mOsm.kg H_2O^{-1}). After 10mins, the perfusion saline was then returned to the control 280mOsm.kg H_2O^{-1} saline and the data recorded for a subsequent 10mins. For cells isolated into 380mOsm.kg H_2O^{-1} DMEM, chondrocytes were perfused with a control saline of 380mOsm.kg H_2O^{-1} for 2mins and then the saline switched to deliver a hypo-osmotic stimulus (380mOsm.kg H_2O^{-1} to 280mOsm.kg H_2O^{-1}). After 10mins, the perfusion saline was then returned to the control 380mOsm.kg H_2O^{-1} saline and the data recorded for a subsequent 10mins.

In response to the decrease in extracellular osmolality, there was an increase in cell volume as indicated by a decrease in 358nm fluorescence. Some chondrocytes subsequently performed RVD. For chondrocytes isolated into 280mOsm.kg H_2O^{-1} DMEM, 58.1 ± 16.6 % of cells showed RVD compared to 81.6 ± 8.9 % of cells isolated into 380mOsm.kg H_2O^{-1} DMEM; no significant difference ($p=0.29$; $n=7$, $N=211$). This data would seem to suggest that a decrease in the isolation osmolality stimulated more cells to respond to a hypo-osmotic challenge with RVD. Further analysis of the data revealed that cells isolated from one joint into 380mOsm.kg H_2O^{-1} DMEM only 25% responded with RVD compared to 77 % & 71 % for the other two joints in this

experimental group. As means and s.e.ms are calculated per joint, this decreased the overall percentage of cells responding value.

After 10mins in the hypo-osmotic saline, the saline was then switched to deliver a hyper-osmotic challenge (by returning to the initial osmolality), resulting in an increase in cellular fluorescence. Despite robust RVD, the 'post RVD-RVI' protocol did not significantly increase ($p>0.05$) the number of chondrocytes showing RVI. For chondrocytes isolated into 380mOsm.kg H_2O^{-1} DMEM, 1.72 ± 0.89 % of cells underwent post RVD-RVI (n=3,N=107) compared to 4.36 ± 1.81 % of chondrocytes isolated into 280mOsm.kg H_2O^{-1} DMEM (n=4, N=104; Table 4.1).

These data show that in response to a hyper-osmotic challenge very few chondrocytes regulated their volume by the process of RVI. Isolating the chondrocytes into a medium of lower osmolality (280mOsm.kg H_2O^{-1}), and the post RVD-RVI protocol, did not increase the number of cells responding. This would therefore suggest that the majority of freshly-isolated chondrocytes do not volume regulate by RVI in response to a direct hyper-osmotic challenge or via the 'post RVD-RVI' protocol.

Together, these data would suggest that irrespective of the isolation osmolality, the majority of chondrocytes do not have the capacity for RVI in response to either a direct hyper-osmotic challenge or the 'post RVD-RVI' protocol.

	Experimental Group (mOsm.kg H_2O^{-1} DMEM)	RVI (% of cells responding)	statistical Difference
(A)	RVI in chondrocytes isolated into 380	6.5 ± 4.15 (n=5, N=86)	N/S
(B)	RVI in chondrocytes isolated into 280	5.26 ± 5.26 (n=3, N=52)	N/S
(C)	Post RVD-RVI in chondrocytes isolated into 380	1.72 ± 0.89 (n=3, N=107)	N/S
(D)	Post RVD-RVI in chondrocytes isolated into 280	3.62 ± 1.27 (n=4, N=104)	N/S

Table 4.1. RVI and Post RVD-RVI by isolated chondrocytes.

Chondrocytes were isolated from bovine articular cartilage and incubated with fura-2 AM (5μM; 30mins). The capacity for RVI measured in response to (A) a direct hyper-osmotic challenge (380mOsm.kg H_2O^{-1} to 540mOsm.kg H_2O^{-1}); (B) a direct hyper-osmotic challenge with cells isolated into a lower osmolality (280mOsm.kg H_2O^{-1} to 420mOsm.kg H_2O^{-1}); (C) Post RVD- RVI (380mOsm.kg H_2O^{-1} to 220mOsm.kg $H_2O^{-1}$to 380mOsm.kg H_2O^{-1}) and (D) Post RVD-RVI (280mOsm.kg H_2O^{-1} to 140mOsm.kg $H_2O^{-1}$to 280mOsm.kg H_2O^{-1}) by fluorescent microscopy. For chondrocytes isolated into 380mOsm.kg H_2O^{-1} (control group) very few cells demonstrated RVI and the number of responding cells was not increased by using a lower isolation osmolality or by the post RVD-RVI protocol. Data are expressed as mean ± s.e.m and the number of joints (n) and cells (N) shown for each experimental group. When compared, there was no significant difference ($p>0.05$; Student's t-test) between the experimental groups as indicated in the last column.

4.2.4 Changes in $[Ca^{2+}]_i$ Associated with an Increase in Extracellular Osmolality.

In bovine articular chondrocytes, it has previously been shown that a decrease in extracellular osmolality initiated a transient rise in $[Ca^{2+}]_i$ mediated by a gadolinium-sensitive ion channel (Erickson *et al.*, 2001; Yellowley *et al.*, 2002). As it is currently unknown if an increase in extracellular osmolality influences $[Ca^{2+}]_i$ in freshly-isolated bovine articular chondrocytes this was studied by fluorescent microscopy in response to a 43% hyper-osmotic challenge. Chondrocytes were incubated with fura-2 AM (5μM; 30mins; 37°C) and experiments performed in parallel with the volume experiments as previously described (see Materials and Methods). A cell was deemed to show a change in $[Ca^{2+}]_i$ (here we just examined changes in intracellular calcium and did not quantify the data as the relationship as this required an accurate Kd, and information in the free and bound forms of fura-2 at 340nm and 380nm) if there was at least a 10 % rise over basal levels (see Materials and Methods).

In response to a direct hyper-osmotic challenge, 52.88 ± 11.48 % of chondrocytes isolated into 380mOsm.kg H_2O^{-1} DMEM responded with a rise in $[Ca^{2+}]_i$ (n=5, N=86). This was significantly reduced ($p<0.01$; Student's t-test) by decreasing the isolation osmolality to 280mOsm.kg H_2O^{-1} where only 4.39 ± 4.39 % of cells responded (n=3, N=52; Fig. 4.5a). It was then tested so see if there was a correlation between the changes in $[Ca^{2+}]_i$ and the capacity for RVI. There were no significant differences (for both isolation osmolalities) in the maximal $[Ca^{2+}]_i$ rise between cells that did and those that did not show RVI although the maximal rise in $[Ca^{2+}]_i$ was significantly decreased ($p<0.05$) in the chondrocytes isolated into 280mOsm.kg H_2O^{-1} DMEM (Fig 4.5b). Of

the 13 cells that did show RVI when isolated into 380mOsm.kg H_2O^{-1} DMEM, 7 underwent RVI without a rise in $[Ca^{2+}]_i$ and 6 with a rise in $[Ca^{2+}]_i$. Only two cells in the 280mOsm.kg H_2O^{-1} DMEM underwent RVI and this was completely independent of a rise in $[Ca^{2+}]_i$.

In response to the post RVD-RVI protocol, a rise in $[Ca^{2+}]_i$ was also evident in some cells. In response to the hyper-osmotic challenge, a transient rise in $[Ca^{2+}]_i$ was exhibited by 60.25 ± 14.20 % of chondrocytes isolated into 380mOsm.kg H_2O^{-1} DMEM and 61.73 ± 13.09 % of chondrocytes isolated into 280mOsm.kg H_2O^{-1} DMEM. Unlike the maximal rise in $[Ca^{2+}]_i$ observed in response to a direct hypo-osmotic challenge, there was no attenuation as a result of a lower isolation osmolality. When correlating the capacity for RVI with the rise in $[Ca^{2+}]_i$, of the 2 cells that did show RVI (when isolated into 380mOsm.kg H_2O^{-1} DMEM) neither exhibited a rise in $[Ca^{2+}]_i$ rise. Conversely, in chondrocytes isolated into 280mOsm.kg H_2O^{-1} DMEM, of the 5 cells that exhibited RVI 2 performed RVI with an associated rise in $[Ca^{2+}]_i$ and in the other 3 chondrocytes intracellular calcium remained unchanged.

These data show that an increase in extracellular osmolality initiated a transient rise in $[Ca^{2+}]_i$ that did not appear to correlate with the capacity for RVI. Isolation into 280mOsm.kg H_2O^{-1} significantly attenuated the maximal rise in $[Ca^{2+}]_i$ and the percentage of chondrocytes responding. Interestingly, the 'post RVD-RVI' protocol (at both isolation osmolalities) restored the percentage of chondrocytes showing a rise in $[Ca^{2+}]_i$ where there was no significant difference between the two experimental groups.

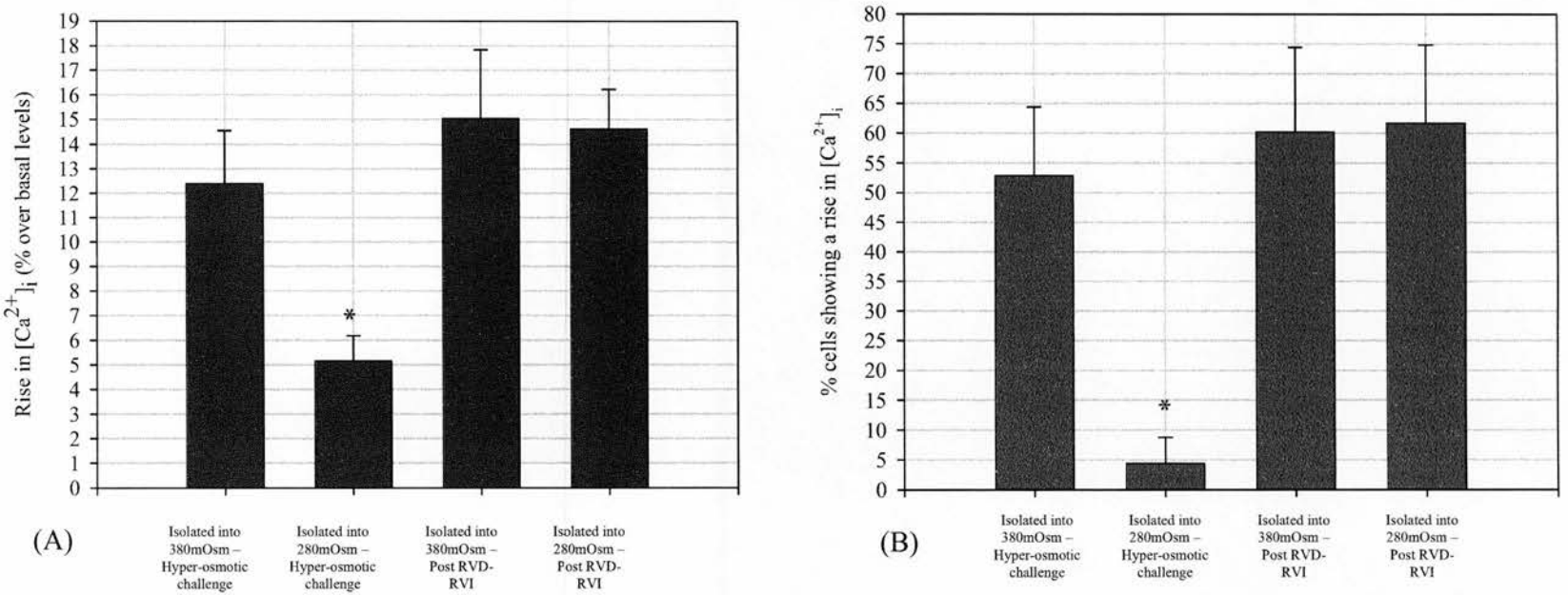


Figure 4.5. ***Changes in $[Ca^{2+}]_i$ in response to hyper-tonicity.***

Changes in $[Ca^{2+}]_i$ were studied in freshly-isolated chondrocytes in response to (A) a direct hyper-osmotic challenge and (B) the post RVD-RVI protocol. Cells were incubated with fura-2 AM (5μM; 30mins; 37°C) and visualised by fluorescent microscopy. Changes in $[Ca^{2+}]_i$ were recorded as a ratio of 358:380nm and expressed as a percentage increase over basal levels recording during the perfusion of an iso-osmotic saline. (A) In response to the increase in extracellular osmolality there was a rise in $[Ca^{2+}]_i$ were the maximal rise was significantly attenuated ($p<0.05$; students t-test) by the isolation into 280mOsm.kg H_2O^{-1} but restored by the post-RVD-RVI protocol. (B) The percentage of chondrocytes responding to the hyper-osmotic challenge with a rise in $[Ca^{2+}]_i$ was calculated as cells showing a rise greater than 10 % over basal levels. Isolation into 280mOsm.kg H_2O^{-1} significantly ($p<0.01$; Student's t-test) decreased the number of cells responding however the response was restored by the post RVD-RVI protocol. Data are expressed as mean ± s.e.m; n= 14 joints N= 349 cells.

In summary, the majority of freshly-isolated chondrocytes did not have the capacity for RVI, and the isolation into a medium of lower osmolality or the 'post RVD-RVI protocol did not further stimulate the RVI response (Table 4.1). Both a decrease and an increase in extracellular osmolality initiate a rise in $[Ca^{2+}]_i$ that appeared to be attenuated by isolation in a medium of lower osmolality (Fig 4.5) but restored by the 'post RVD-RVI protocol'. Of the cells that did volume regulate by RVI, there did not appear to be a correlation with the $[Ca^{2+}]_i$ transient.

4.2.5 The NKCC is Expressed by Freshly-Isolated Chondrocytes.

As some chondrocytes were able to volume-regulate in response to a hyper-osmotic challenge, the expression of the NKCC cotransporter was subsequently tested. As previously mentioned (section 1.7.1) the NKCC has been shown to be involved in mediating RVI in various cell types (O'Neill, 1999) and as the presence of a bumetanide-sensitive K^+ has been recorded in chondrocytes (Hall *et al.*, 1996a; 1996b) it was likely that this transporter was expressed. Chondrocytes were isolated into 380mOsm.kg H_2O^{-1} DMEM and cell membrane proteins prepared as previously described (see Appnedix 2). The membrane proteins were subsequently subjected to SDS-gel electrophoresis using gel for 90 mins[5]. The separated proteins were then transferred to nitrocellulose (by an overnight transfer at 4°C) and using a specific mouse monoclonal antibody to the Na-K-2Cl co-transporter (Matskevich & Flatman, 2003) western blot analysis was performed.

Analysis of the Western blots (4 blots were used to enable the calculation of the molecular weight) it was found that the NKCC protein was present in the chondrocyte membrane preparation. A positive control of ferret erythrocyte membrane proteins were used to test the functionality (and cross reactivity) of the antibody (not shown) as the presence of the NKCC had already been shown in this cell type (Matskevich & Flatman, 2003). Two 40μg/ml of chondrocyte membrane protein were ran and analysis on 4 gels

[5] The running and blotting of the Gels was performed by Dr Ioulia Matskevich in Dr Peter Flatman's laboratory.

revealed that the top of the band lay at 211 ± 11.21 kDa and the bottom of the band at 162 ± 3.60 kDa. These molecular weights are consistent with the presence of the co-transporter (Fig. 4.6) and therefore suggest the presence of the NKCC.

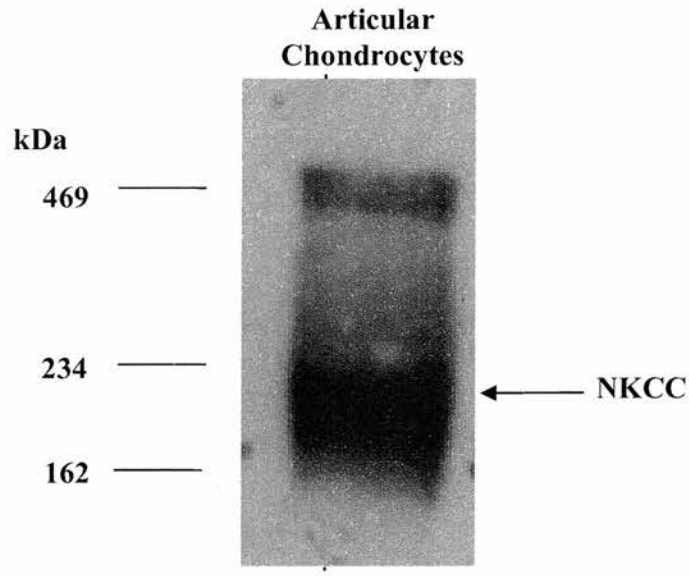


Figure 4.6. Illustrative western blot of chondrocyte membranes.

A membrane preparation was prepared from freshly-isolated chondrocytes and analysis performed by SDS-gel electrophoresis and Western blotting. The blots were probed using a specific antibody to the Na-K-2Cl co-transporter and an average of 4 gels ran to calculate the molecular weight of the resultant bands. The above image is of a single gel where 40μg chondrocyte membrane proteins were ran. The average of 4 blots showed that the top of the band lay at 211 ± 11.21 kDa and the bottom of the band at 162 ± 3.60 kDa. These molecular weights are consistent with the presence of the co-transporter. Data are expressed as mean ± SD and the molecular weights calculated by Dr Ioulia Matskevich. For this analysis, membrane proteins were acquired from 20 joints; N= 4 gels.

4.2.6 RVI in 2D Cultured Chondrocytes.

In section 4.2.3, it was shown that the majority of freshly-isolated chondrocytes were not able to volume regulate in response to a hyper-osmotic challenge. Neither a decrease in the isolation osmolality or the 'post RVD-RVI' protocol stimulated the response despite the capacity for robust RVD. As it has been shown that time in 2D culture alters the chondrocytic phenotype and potentially the expression of membrane channels (section 4.2.4), the capacity for RVI was studied in 2D cultured chondrocytes. Currently it is unknown how time in culture influences the chondrocyte RVI response and therefore this was studied in cultured bovine articular chondrocytes in response to a 43% hyper-osmotic challenge.

4.2.6.1 Confocal images of 2D cultured chondrocytes.

Chondrocytes were isolated from bovine articular cartilage and cultured as previously described (see Materials and Methods). Chondrocytes were subsequently passaged onto 22mm coverslips, incubated with calcein AM (5μM; 30mins; 37°C) and imaged using a Zeiss Axioskop LSM510 upright Confocal Laser Scanning Microscopy (CLSM). To improve image quality a z-step of 90nm was used and all images were averaged 16 times. This high level of acquisition was made possible as each cell was only imaged once or else there would be bleaching of the fluorophore.

Time in culture had a dramatic effect on morphology as shown in fig 4.7. In this image, there are examples of two chondrocytes that had reverted to a fibroblastic[6] morphology and one that had not. To show the extent of the change in chondrocyte morphology, the confocal image is shown as a z-series (a z-series refers to each optical slice acquired by the confocal microscope in the z-plane; Fig. 4.7) and the changes in morphology seen in relation to 'cell height'. From this, it is possible to determine that the fibroblastic chondrocytes was approximately 5.4μm in height whereas the rounded chondrocyte had a height (diameter) of 14.4μm. Taking the image from Fig 4.7 and compiling a projected image (where the images are layered on top of each other), it was possible to see that the fibroblastic cells had formed what appeared to be focal adhesion points to the culture surface (as shown by areas of high fluorescent intensity towards the cell periphery; Fig. 4.8). Interestingly the fibroblastic cells also appeared more granular when compared to the rounded chondrocyte.

One situation that may prevent a chondrocyte from de-differentiating is the lack of attachment to culture surface during time in culture. When reviewing the z-series Fig. 4.7, it is possible to see the base of the differentiated cell does not show in the series until image 5 (3.6μm) up from the culture plastic. This may imply that the cell is either loosely attached (either to another cell or culture plastic) or sitting on top of non-fluorescent debris and therefore may explain how the differentiated morphology appeared to be maintained.

[6] The word fibroblastic is refers to a chondrocyte that has undergone morphological changes and represents the de-differentiated fibroblastic phenotype. It is important to note that no experiments were performed to determine the phenotype of the cell and it is therefore possible that the cells had changed shape while maintaining the chondrocytic phenotype.

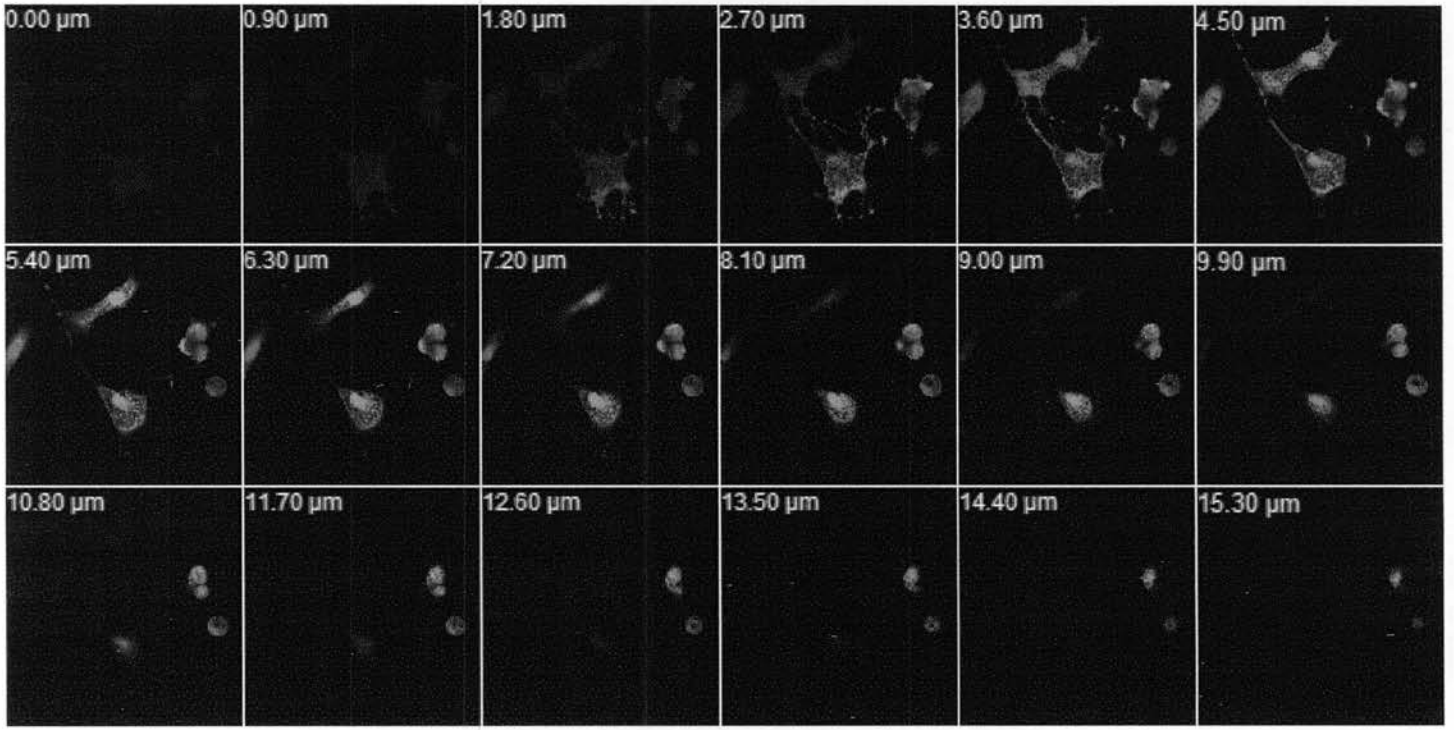


Figure 4.7. Confocal z-series of the previous image of 2D cultured chondrocytes.

Chondrocytes were isolated from full depth bovine articular explants and cultured as previously described. Cells were subsequently incubated with calcein-AM (5µM; 37°C, 30mins) and visualised by Confocal Laser Scanning Microscopy (CLSM). Images were acquired using 90nm z-steps and x16 averaging to increase image resolution. After time in 2D culture, two of the three chondrocytes had reverted to a fibroblastic phenotype and became less spherical and more flattened. From the z-series it is possible to calculate the rough 'height' of the cells. The de-differentiated chondrocytes in this example are approximately 5.4µm in height compared to the approximate height of 14.4µm of the differentiated chondrocyte. The cell that did not de-differentiate does not appear to be attached to the culture plastic.

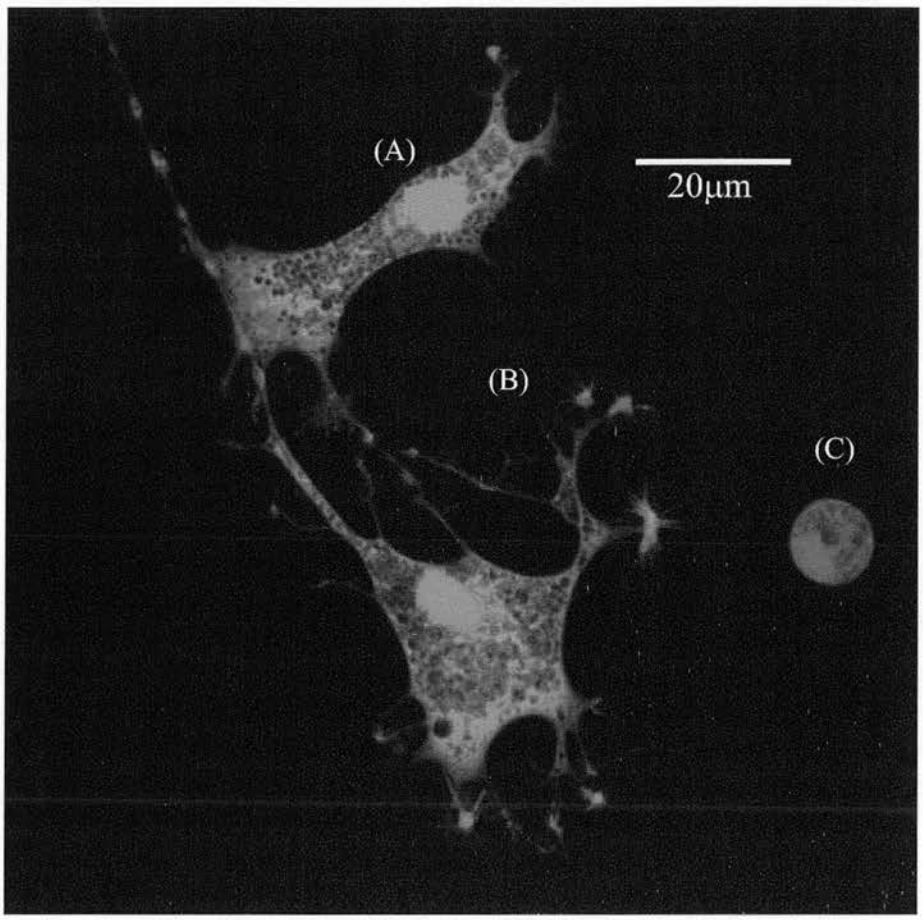


Figure 4.8. Confocal image of cultured chondrocytes.

Chondrocytes were isolated from full depth bovine articular explants and cultured as previously described (see Materials and Methods). Cells were subsequently incubated with calcein-AM (5μM; 37°C, 30mins) and visualised by Confocal Laser Scanning Microscopy (CLSM). The projected image (a process where each image from a single z-plane is viewed as a projected stack) shows two fibroblastic chondrocytes (A & B) and one cell that appeared to have retained a differentiated morphology (C). The cells that have reverted to a fibroblastic morphology (A & B) are less rounded and have developed processes whereas the other cell (C) still resembles the differentiated spherical phenotype.

4.2.6.2 Fluorescent Images of 2D Cultured Chondrocytes

The confocal images shown in section 4.2.6.1 have shown the detailed changes in chondrocyte morphology as a result of 2D culture. To study the capacity for RVI in 2D cultured chondrocytes cells were imaged using the PTI ***Imagemaster™*** system and for comparison images of 2D cultured chondrocytes are shown (Fig. 4.9). Prior to imaging, the cells were passaged onto 22mm autoclaved coverslips and incubated with calcein AM (5μM; 30mins; 37°C). Cells were then transferred to the heated stage (37°C) of a Nikon microscope and imaged as previously described (see Materials and Methods).

After three weeks in 2D culture, the cells had flattened from the spheroidal appearance observed in freshly-isolated chondrocytes and resembled the fibroblastic phenotype (Fig. 4.9a & Fig. 4.9b). In order to study volume regulation in calcein-loaded cultured chondrocytes, images were acquired using a low detector setting as to avoid saturation and a drift in the calcein fluorescence. Using a low detector setting decreased the image detail and therefore unlike the images acquired by CLSM (see Fig. 4.8) the fluorescent microscope images show cellular processes but not as clearly.

Using the PTI ***Imagemaster™*** software, Regions of Interest (ROI's) were drawn around the cells to allow the collection of photometric data (as shown by white boxed regions in Fig.4.9). Data were then collected in response to the hyper-osmotic challenge and transferred to MAX™ and Excel™ for subsequent analysis.

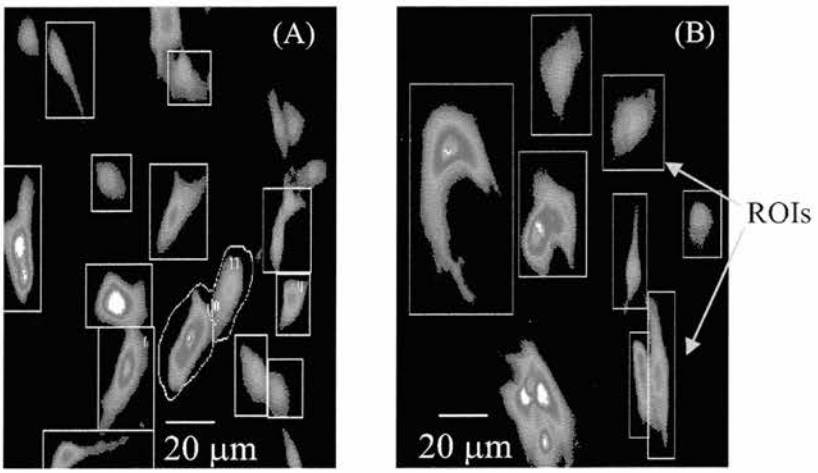


Figure 4.9. Fluorescent images of 2D cultured chondrocytes.

Chondrocytes were cultured for 3 weeks, passaged and incubated with calcein AM (5µM; 37°C, 30mins; see Materials and Methods). The cells (contained with steel rings) were then placed onto a heated stage of a Nikon microscope maintained at 37°C. The cells were visualised through an x40 oil objective lens and the calcein excited at 495nm (recorded emission of 525nm) using a PTI Imagemaster™ system. (A & B) After time in 2D culture, the cells had flattened from the spherical differentiated phenotype (as seen in in-situ and in freshly-isolated cells) and resembled a fibroblastic phenotype. To enable the collection photometric data suitable for the study of RVI, a low detector setting was used and hence it was not possible to visualise cell processes. Regions of interest (ROIs) were drawn around the cells (as shown by numbered white boxes) and the photometric data collected within. Images are shown as raw acquisition images without modification; n=cells isolated from 1 joint.

4.2.7 Change in the Capacity for RVI after 2D culture.

It has previously been shown in freshly-isolated chondrocytes that 6.25 ± 4.15 % of cells responded to a hyper-osmotic deformation with RVI (Table 4.1). As time in 2D culture has been shown to influence chondrocyte morphology (Benya & Shaffer, 1981; Benya *et al.*, 1981; Benya & Shaffer, 1982) the RVI response was subsequently tested. Chondrocytes were isolated from full depth bovine articular cartilage explants and cultured as previously described (see Materials and Methods). After 3 weeks in culture, cells were passaged onto 22mm coverslips, incubated with calcein-AM (5μM; 30mins; 37°C) and RVI studied in response to a 43 % hyper-osmotic challenge (see Materials and Methods).

In response to the osmotic challenge, there was an increase in cellular fluorescence indicating a decrease in cell volume. At maximal cell shrinkage, there was an increase in fluorescence of 3.5 ± 0.35 % which was significantly less ($p<0.05$) than the 9.8 ± 1.2 % observed in freshly-isolated chondrocytes; (section 4.2.3; n=9, N=190). This difference in the maximal change in fluorescence can potentially be explained by the measurement of changes in cell volume. In freshly-isolated cells, the focal plane is through the middle of the cell and encompasses a very small part of cell body. Therefore, during cell shrinkage, the amount of fluorophore is concentrated within the optical plane and the fluorescence increases in intensity. Conversely, in 2D cultured cells, the focal plane encompasses more of the cell and even though the cell may swell to the same extent the actually amount of fluorophore being 'moved' into the optical plane is less. Therefore, when compared to freshly-isolated chondrocytes the extent of

the fluorescent change is less. It has been commented that measurement in cell volume in 2D cultured chondrocytes is difficult and that the use of freshly passaged cells provide a more uniform change in cellular fluorescence (Yellowley *et al.*, 2002). See discussion (section 4.4) for more details.

As seen in freshly-isolated chondrocytes some cells then underwent RVI. Data were plotted and the rate of RVI (expressed as a t½) calculated by linear regression (r^2>0.9; time taken for volume to be recovered by 50%; see Materials and Methods). It was found that the t½ was 3.13 ± 0.49 % mins which was significantly less ($p<0.05$; Student's t-test) than 6.1 ± 0.6 mins recorded in freshly-isolated cells. This difference can most likely be explained by the fact that there was less apparent swelling in cultured cells and therefore the extent of RVI required less. When comparing the rate (expressed as fluorescence units per minute $x10^3$; $Fu.min^{-1}$) there was no significant difference ($p>0.05$) with rates of -6.81 ± 1.031 $Fu.min^{-1}$ and -7.48 ± 0.785 $Fu.min^{-1}$ between cultured isolated and freshly isolated cells respectively; (n=9, N=190).

When calculating the percentage of cells that showed RVI (cells were deemed to have shown RVI if at the end of the experimental period volume had been recovered by at least 50%), there was a significant increase ($p<0.001$) in the number of cells responding when compared to freshly-isolated cells. As shown in Figure 4.10, in freshly-isolated chondrocytes, 6.25 ± 4.15 % of cells showed robust RVI and this was increased to 54.86 ± 5.41 % after three weeks in 2D culture; (n=9, N=190).

To study the role of the NKCC in cultured bovine articular chondrocytes RVI, experiments were performed in the presence of 75μM bumetanide (inhibitor of the Na-K-2Cl; (Flatman, 2002). There was no significant difference in cell shrinkage (3.06 ± 0.2 %), the rate of RVI ($t½$ = 2.29 ± 0.51; $Fu.Min^{-1}$) or the percentage of cells responding (69.74 ± 3.60 %) when compared to control cells (n=3, N=54). These data would seem to imply that the NKCC is not involved in cultured chondrocyte RVI. To further elucidate the mechanism of RVI, a detailed inhibitor study needs to be performed.

These data show that some cultured bovine articular chondrocytes have the capacity for RVI and that the percentage of cells responding increased after 3 weeks in 2D culture when compared to freshly-isolated cells. When comparing the rate, the extent of cell swelling and recovery between freshly-isolated chondrocytes and 2D cultured chondrocyte, there was no significant difference. Preliminary data using 75μM bumetanide would indicate that RVI in cultured bovine articular chondrocytes was not mediated via the NKCC co-transporter.

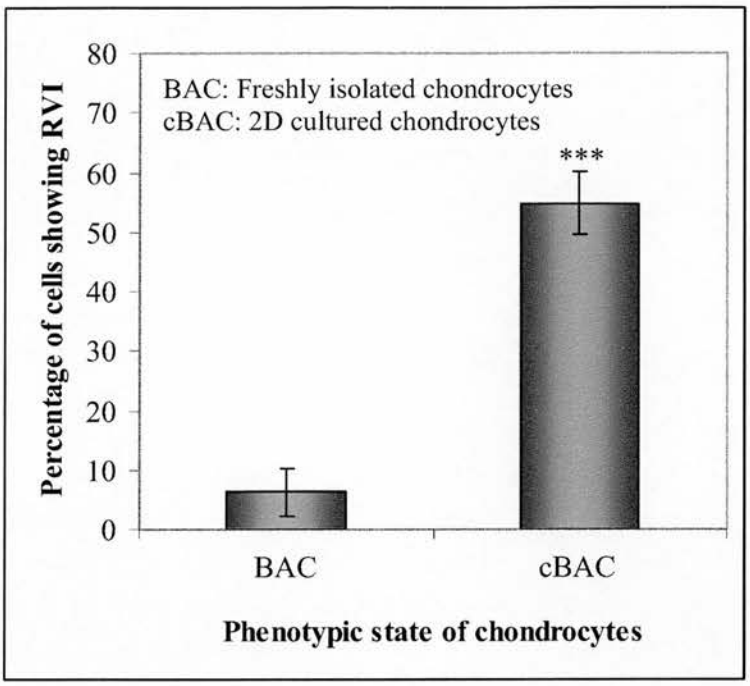


Figure 4.10. The capacity for RVI changes after time in 2D culture.

*Chondrocytes were isolated from full depth bovine articular cartilage explants and cultured in 2D for 3 weeks (see Materials and Methods). Cells were then passaged onto 22mm coverslips, incubated with calcein AM (5μM; 30mins; 37°C) and RVI studied by fluorescent microscopy in response to a 43% hyper-osmotic challenge. In response to a hyper-osmotic challenge, 14.25 ± 6.25 % of freshly-isolated chondrocytes (BAC's) were able to regulate back to their initial volume by RVI. After 2 weeks in culture (cBAC) the percentage of cells exhibiting RVI increased to 54.86 ± 5.41 % ($p<0.001$; Student's t-test). Data are expressed as percentage cells showing greater than 50% RVI at the end of the 10 minute experimental period. For freshly-isolated chondrocytes, n=5 joints; N= 86 cells. For cultured chondrocytes, n=9 joints, N=190 cells. A significant difference (calculated by a Student's t-test) of $p<0.001$ is indicated by ***.*

4.3.0 Results Summary.

The data presented in this chapter have shown that the majority of freshly-isolated chondrocytes do not have the capacity for RVI despite the expression to the NKCC cotransporter (Table 4.1; Fig. 4.6). A decrease in the isolation osmolality (from 380mOsm.kg H_2O^{-1} to 280mOsm.kg H_2O^{-1}) or the 'post RVD-RVI' protocol did not stimulate the response. The increase in extracellular osmolality resulted in a transient rise in $[Ca^{2+}]_i$ that did not appear to correlate with the capacity for RVI.

Conversely, after two weeks in 2D culture, significantly more chondrocytes were able to respond to the hyper-osmotic challenge with RVI (Fig. 4.10). Preliminary data suggests that the RVI was insensitive to 75μM bumetanide and therefore may not be mediated by the NKCC cotransporter.

4.4.0 Chapter Discussion.

The data presented in this chapter have shown that freshly-isolated chondrocytes do not have the capacity for RVI despite the expression of the NKCC (Table 4.1; Fig. 4.6). Conversely, after time in 2D culture there was a significant increase in the percentage of cells showing RVI (Fig.4.10). The increase in extracellular osmolality initiated a transient rise in $[Ca^{2+}]_i$ that did not appear to correlate with the capacity for RVI when studied in freshly-isolated chondrocytes.

In response to a 43% hyper-osmotic challenge, the majority of chondrocytes (~92%) did not have the capacity for RVI. Furthermore, it was not possible to further stimulate the RVI response by the 'post RVD-RVI' protocol or by reducing the isolation osmolality. When tested, it was shown that chondrocytes express the NKCC cotransporter although its the functionality was not shown in this piece of work. Of interest is the fact that in response to 2D culture the capacity for RVI is developed (or realised) and may relate to the phenotype, morphology or organisation of the actin cytoskeleton all known to change in during time in 2D culture (Benya *et al.*, 1981; Benya & Shaffer, 1982; Benya *et al.*, 1988; Idowu *et al.*, 2000; Langelier *et al.*, 2000; Zwicky & Baici, 2000a).

The lack of RVI observed in the majority of chondrocytes in response to a direct increase in extracellular osmolality was not surprising, as this has been observed in many other cell types (O'Neill, 1999) although it is present in some cell types including cultured human skin fibroblasts (Dallasta *et al.*, 1994). It was shown that RVI (mediated by sodium-dependent amino acid transport) was slow to activate (despite a chronic challenge of 300-400mOsm) with no apparent recovery until 20mins post-osmotic

challenge with complete RVI in 3 hours. It was possible that the culturing of the cells may have influenced the capacity for RVI, and interestingly as length of the time course was 3 hours, this may suggest it would have been possible to detect RVI in chondrocytes if the time period was increased. To resolve this, longer time course experiments would need to be performed. Conversely, it has been commented that most cells are capable of 'post RVD-RVI' (Errington *et al.*, 1997; Hoffmann & Pedersen, 1998; O'Neill, 1999) and interestingly bumetanide-sensitive 'post RVD-RVI' has been observed in porcine chondrocytes *in situ* (Errington *et al.*, 1997; Hoffmann & Pedersen, 1998; O'Neill, 1999). This raises the question as to why in this study no significant 'post RVD-RVI' was observed. Possibilities could include: (1) cell damage, (2) interaction with the ECM, (3) ion gradients and (4) the experimental period was too short. Each of these will be now be discussed in turn.

If chondrocytes were damaged during the isolation from the ECM, this may explain the lack of RVI in freshly-isolated chondrocytes and the recovery seen in 2D cultured cells. Arguments against this would be that the freshly-isolated chondrocytes had the capacity for RVD (Fig. 4.3) and therefore it would be fair to suggest that the mechanism involved in the 'sensing' and responding to changes in extracellular osmolality were functional (Bush & Hall, 2001b; Yellowley *et al.*, 2002). It could be suggested that the mechanisms for sensing shrinkage and swelling are different and therefore this alone is not a particularly strong argument. Both NKCC and Na^+-K^+ pump activity, stimulated by hyper-osmolality have been recorded in isolated bovine articular chondrocytes, suggesting that these were not damaged upon release from the cartilage matrix (Hall *et al.*, 1996b).

Another possibility to account for the differences observed between the *in vivo* porcine experiments and *in vitro* data presented here, could be that chondrocytes may require a signal/stimulus from the ECM to initiate the RVI response. Chondrocytes form integrin complexes that facilitate the transduction of mechanical forces from the extracellular environment to the cell (Millward-Sadler 1999). These are dependent upon a polymerised F-action cytoskeleton and inhibited by a pre-incubation with cytochalasin D (Wright *et al.*, 1996; Millward-Sadler *et al.*, 1999; Millward-Sadler *et al.*, 2000b). It has been shown that that the activation of the NKCC is sensitive to a polymerised F-actin cytoskeleton (Matthews *et al.*, 1994; Matthews *et al.*, 1997), and therefore it is possible that isolation from the ECM inhibits NKCC activation by disruption of integrin signalling complexes or by a altering the organisation of the F-actin cytoskeleton. As NKCC activity is stimulated by the depolymerisation of the F-actin cytoskeleton (Matthews *et al.*, 1997), the NKCC inactivation could be a result of the net increase of F-actin as shown in response of a hyper-osmotic challenge (Rizoli *et al.*, 2000; Di Ciano *et al.*, 2002; Guilak *et al.*, 2002; Koffer *et al.*, 2002; Lewis *et al.*, 2002). An argument against this would be that cell shrinkage has been shown to activate the NKCC in many other cell types including human epithelial T84 cells (Matthews *et al.*, 1998). Furthermore, in chondrocytes an increase in cellular F-actin has not been observed in response to an increase in extracellular osmolality (Guilak *et al.*, 2002).

The ionic environment of cartilage is very different from that of most other tissues, with raised levels of cations and a decreased level of anions (Maroudas 1980). When comparing the ion concentrations used in the experimental saline to that *in situ* there is an increased level of extracellular Cl^- (~190mM compared to ~20mM in the deep zone;

Maroudas 1980) and a decreased level of extracellular Na^+ (~190mM compared to ~350mM in the deep zone; Maroudas 1980). It is therefore possible that these changes in extracellular ion concentration influence the activity of the NKCC cotransporter and thus prevent RVI. One possibility would be a decrease in extracellular $[Na]_o$ results in an decrease in intracellular pH due to the reduced activity of the Na^+-H^+ exchange. This change in pH may then consequently influence NKCC activity. It has been shown in an oocyte expression system that a decrease in pH reduced NKCC activity although this was particular to the NKCC2 isoform and not the NKCC1 (Bergeron *et al.*, 2003). The expression of the NKCC1 isoform has been shown in chondrocytes (see section 4.2.5; (Trujillo *et al.*, 1999) but as of yet the expression of the NKCC2 is unknown. Conversely an increase in $[Cl^-]_o$ may also attenuate the activation of the NKCC cotransporter potentially mediated by a 'leak' into the cell thus raising intracellular chloride and inhibiting NKCC activation by Cl^- sensitive phosphorylation (Breitwieser *et al.*, 1996). It has been commented that NaCl induced cell shrinkage raises $[Cl^-]_i$ and this may have been the case here (Waldegger *et* al., 1998). Should $[Cl^-]_i$ increase due to time in the culture media and during time in the experimental saline, then this may impede the activation of the NKCC.

As well as $[Cl^-]_i$ influencing the activity of the NKCC kinase and subsequent activation it is also thought that $[Cl^-]_i$ may have a direct affect on the cotransport (Flatman 2002). It has been shonwn in Ehrlich ascites cells and in salivary acinar cells that $[Cl^-]_i$ blocks net influx in response in an osmotic challenge and in other cell types it has been suggested that the 'resting' chloride concentration inhibits activity at steady-state volume (O'Neill 1999). It has been suggested by Jiang *et al.*, 2001 that high $[Cl^-]_i$

blocks outward translocation of the 'empty' cotransport by binding directly to the cotranporter although the exact mechanism is still unclear (Jiang *et al.,* 2001). If this were found to be correct then it has been suggested as a simple method of regulating intracellular chloride concentration via a negative-feedback loop. To examine the effects of these, the experiments could be repeated adjusting the levels of Cl^- and Na^+ concentrations to match those *in situ* perhaps using NO_3^- replacement for Cl^- and NMDG for Na^{2+}.

RVI mediated by the NKCC cotransporter has been shown to be sensitive to levels of intracellular chloride where high levels of $[Cl^-]_i$ inhibit the phosphorylation and subsequent initiation of the NKCC mediated RVI (Breitwieser *et al.*, 1996; O'Neill, 1999; Flatman, 2002). In response to a direct hyper-osmotic challenge, chondrocyte RVD is mediated by the loss of intracellular osmolytes including K^+, Cl^- and taurine (Hall *et al.*, 1988; Hall, 1994, 1995; Hall *et al.*, 1996b; Hall & Bush, 2001) and recently it was shown that the major osmolyte was Cl^- (Yellowley *et al.*, 2002). Therefore, this decrease in $[Cl^-]_i$ would seem favourable to the activation of the NKCC in response to the 'post RVD-RVI' protocol. In this study the lack of RVI may not be attributed to the failure of NKCC activation and may suggests that the NKCC is regulated outwith the methods previously mentioned or not involved in mediating RVI in isolated bovine articular chondrocytes.

The remaining reason that significant RVI was not observed could be that the experimental period was too short. The lack of significant robust RVI has been shown in porcine articular chondrocytes where after a 10min experimental period volume was

only recovered by ~10% (Erickson *et al.*, 2001). Furthermore, In cultured human skin fibroblasts, it was shown that RVI was slow and took 3 hours to mediate almost a complete recovery in volume (Dallasta *et al.*, 1994). It is therefore possible that the RVI response in chondrocytes may be slow to initiate and in mediating the recovery of volume.

The most significant finding of this study is that after time in 2D culture, chondrocytes develop the capacity for RVI in response to a direct hyper-osmotic challenge. Furthermore, preliminary data would suggest that the RVI was bumetanide-insensitive and therefore probably not mediated by the NKCC cotransporter. This would therefore suggest that another mechanism must be involved in the 2D cultured chondrocyte RVI response. One possibility is the Na^+-H^+ antiport coupled to the HCO_3^-/Cl^- exchange. Chondrocytes have been shown to expresses a functional Na^+-H^+ antiport (NHE; (Wilkins *et al.*, 1995b; Wilkins *et al.*, 1996; Browning *et al.*, 1999; Trujillo *et al.*, 1999) and in conjunction with a functional Na^+ dependent- HCO_3^-/Cl^- exchange this is able to mediate RVI (Dascalu *et al.*, 1996; O'Neill, 1999). In bovine articular chondrocytes, the expression of the HCO3-/Cl- exchange has not been confirmed although it has been demonstrated in cultured avian articular chondrocytes (Dascalu *et al.*, 1993). It is possible that during time in culture the expression of the HCO_3^-/Cl^- exchanger is induced and this in conjunction with the fact that hyper-osmolality has been shown to induce NHE activity may bring about the capacity for RVI (Wilkins *et al.*, 1995a). An argument against this would be that the experiments were performed using an artificial HEPES pH buffered saline and therefore there would be little (if any) HCO_3^-/Cl^- activity. If this were the case then another possibility would include the sodium-

dependent amino acid transporter, although this is me be rather slow [Dallasta *et al.*, 1994; Wilkins *et al.*, 1995b) and the RVI observed here was robust (under 10mins).

The simplest explanation to explain the lack of RVI is that it is not physiologically necessary and therefore chondrocytes do not have to capacity to volume regulate in response to a hyper-osmotic challenge. This could be due to the diurnal cyclic hydration and dehydration of cartilage where during the day (standing) cartilage is slowly compressed whereas at night (when laying down) the compressive forces are removed and cartilage rehydrates. It has been shown that hydrostatic pressure results in the inactivation of the both the NKCC and the Na^+-K^+ pump where the activity is restored upon pressure release (Hall, 1999). This would seem to suggest that if RVI mediated by either of these transport systems then it is effectively 'turned off' in response to compressive load. Furthermore, it has been suggested that the efficacy of the chondrocyte NKCC is low, (9.6 nmol.10^6 cells^{-1}.h^{-1} compared to 84.9 nmol.10^6 cells^{-1}.h^{-1} recorded for the Na^+- K^+ pump; (Hall *et al.*, 1996b) and therefore it is possible that this is just not enough to mediate an effective RVI response in chondrocytes. If however RVI was mediated by the NHE, whose activity is stimulated by hydrostatic pressure and hyper-osmolality (Browning *et al.*, 1999) then another explanation is required to explain the lack of RVI in this study.

In conclusion, it does not appear the freshly-isolated chondrocytes have the capacity for RVI or 'post RVD-RVI' in response to an increase in extracellular osmolality by NaCl addition. Conversely, after two weeks in 2D culture, the capacity for RVI is realised and preliminary data would suggest that it is not mediated by the NKCC. The exact

mechanism of 2D cultured chondrocyte RVI is yet to be elucidated although it is possible that time in culture altered the membrane transporters required to mediate the response. Furthermore, It is possible that after time in culture chondrocyte begin to synthesise a pericellular matrix thus making a localised micro-environment more conducive to RVI. The initiation of RVI has been shown to be closely linked to the cytoskeleton, it is possible that time following isolation is required to restore the signalling pathway. To further understand RVI in chondrocytes, *in situ* confocal studies relating the capacity for hyper-osmotic and 'post RVD-RVI' to the zone of cartilage would be useful as it is conceivable that the capacity for RVI may differ between the three main zones of cartilage.

Finally, it has been shown that chondrocyte volume increases with OA above that predicted by free swelling as a result of cartilage hydration (Bush & Hall, 2003). One possible mechanism that could mediate this is the up regulation of NKCC activity or any other RVI transporters. An increase in activity would result in the influx of ions, and consequently osmotically obliged water therefore increasing cell volume. Therefore, correlating NKCC activity to cartilage degeneration may provide an insight into why chondrocytes swell and a further insight into the degenerative state. It has already been shown that there is no increase in NKCC expression associated with cartilage degeneration (Trujillo *et al.*, 1999) although the paper employed no method of quantification and therefore a repeat of these experiments calculating NKCC expression would be prudent.

The role of the actin cytoskeleton in chondrocyte RVD

5.1.0 Chapter Introduction

In chondrocytes, the F-actin cytoskeleton is a potent regulator of the chondrocytic phenotype and has been implicated in signal transduction in response to mechanical stimulation (Benya *et al.*, 1988; Wright & Salter, 1996; Millward-Sadler *et al.*, 1998b; Millward-Sadler *et al.*, 1999; Salter *et al.*, 2001). In freshly isolated and *in situ* chondrocytes the F-actin cytoskeleton is organised cortically, just below the plasma membrane with little across the cell body (Langelier *et al.*, 2000). Converely, in 2D cultured chondrocytes the organisation of the F-actin cytoskeleton changes, resulting the the formation of stress fibres that span the cell (Zwicky *et al.*, 2000). The organisation of the F-actin cytoskeleton is sensitive to changes in extracellular osmolality where in most cell types a decrease in osmolality results in the depolymerisation of the cytoskeleton and an increase in osmolality increases cellular F-actin (Hoffman 2000; Pedersen *et al.*, 2001).

Overhydration (and the subsequent decrease in cartilage osmolality) observed is one of the first visible symptoms of OA (Grushko *et* al., 1989; Stockwell 1991; Bank *et al.*, 2000). These changes will therefore affect cell volume and volume measurements taken from *in situ* human articular chodrocyes, found cell volume increased with pathology (Bush & Hall 2003). In chondrocytes, decrease in extracellular osmolality results in actin depolymerisation (Erickson *et al.*, 2003) where conversely an increase in extracellular osmolality interestingly had no affect on cellular F-actin (Guilak *et al.*, 2002). Therefore, as changes in osmolality influence the actin cytoskeleton it is possible that there changes have a role in volume regulation. In other cell types, it has been

shown that RVD is dependent upon a polymerised F-actin cytoskeleton. For example in Ehrlich Ascites Tumour cells (EAT cells), RVD is mediated by the activation of Cl^- and K^+ channels and can be inhibited by cytochalasin B (Pedersen *et al.*, 2001). Conversely, as seen in both HL-60 cells and human peripheral blood neutrophils, RVD is unaffected by F-actin depolymerisation (Downey *et al.*, 1992; Downey *et al.*, 1995). This therefore suggests that the role of the actin cytoskeleton on the RVD is dependent upon cell type and possibly the phenotype of the cell.

In bovine articular chondrocytes, the role of an intact F-actin cytoskeleton in chondrocyte volume regulation is currently unknown. In both freshly-isolated and 2D cultured bovine articular chondrocytes, RVD is mediated by an 'osmolyte channel', whose pharmacology remains to be elucidated but yet can be inhibited by REV 5901 (Bush & Hall, 2000, 2001a, 2001b). Therefore, it is possible that the RVD response could involve the actin cytoskeleton. There are numerous ways in which the F-actin cytoskeleton could be involved in RVD including: (1) The direct regulation of the channel as seen in the NKCC cotransporter (Matthews *et al.*, 1997); (2) Forming part of the volume sensor complex involved in 'sensing' changes in osmolality (Hoffmann, 2000; Al-Habori, 2001) or (3) in the translocation of an intracellular channel store to the plasma membrane (Emma *et al.*, 1998). Conversely, it is possible that the observed changes in F-actin re-organisation in response to change in osmolality are not involved in volume regulation and may be implicated in another cellular response.

In this study, the capacity for RVD in freshly-isolated and 2D cultured bovine articular chondrocytes was studied in the presence of using Latrunculin-B (a potent disrupter of

the actin cytoskeleton; (Wakatsuki *et al.*, 2001). Latrunculin B was used as an inhibitor of the actin cytoskeleton as it is more specific to actin and when compared to the cytochalasin family of inhibitors (Table 1.3) had the least adverse 'side effects'. RVD was measured by fluorescent microscopy using both fura-2 (for freshly-isolated cells) and calcein (for 2D cultured cells) in response to a 43% hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) by perfusion. The organisation of the actin cytoskeleton was visualised on fixed cells by confocal microscopy with the polymerised F-actin labelled using phalloidin-FITC and monomeric G-actin was labelled using DNase-Texas Red. Furthermore, as bovine articular chondrocyte RVD can be inhibited by 50μM REV 5901 (Bush & Hall, 2000; Hall & Kerr, 2000), the effect on the polymerised F-actin cytoskeleton was also studied.

The use of real time fluorescent imaging and confocal microscopy provided a powerful tool in linking a dynamic cellular process to the structural organisation of the cell. This confers confidence in the inhibitors used and strengthens any experimental findings. Furthermore, the advancements in confocal imaging allow for the detailed visualisation of the actin cytoskeleton that may have been missed by just using fluorescent microscopy.

Hypothesis to be tested:

RVD in articular chondrocytes is dependent upon an intact actin cytoskeleton

5.2.0 Results

The capacity for RVD was studied by fluorescent microscopy using a PTI ***Imagemaster™*** system and the morphology of the actin cytoskeleton studied by CLSM. As the visualisation of the actin cytoskeleton required the use of fixed cells, these two techniques used in conjunction allowed the relation of a dynamic cell process (volume regulation) to fixed 'snapshot' images showing actin organisation. For the measurement of RVD, freshly-isolated chondrocytes were incubated with fura-2 AM and 2D cultured chondrocytes were incubated with calcein AM. The actin cytoskeleton was labelled using phalloidin-FITC (binds to polymerised F-actin; green) and DNase-Texas Red (binds to monomeric G-actin; red).

5.2.1 Images of Chondrocytes

5.2.1.1 Freshly Isolated Chondrocytes

When loaded with fura-2, and viewed through a single optical plane the chondrocytes appeared spheroidal with some heterogeneity in apparent cell size (Fig. 5.1a). The differences observed in cell size could be attributed to the zone from which the cells were derived. When comparing the morphology of control and Latrunculin B treated chondrocytes, there did not appear to be any differences as in both experimental groups the cells appeared rounded (Fig. 5.1b).

5.2.1.2 2D Cultured Chondrocytes

After 3 weeks in 2D culture (see Materials and Methods), the chondrocytes had flattened from the spheroidal shape (observed previously Fig. 5.1a) and appeared to resemble the fibroblastic phenotype. The cells were less rounded and approximately 3-5μm in height (predicted using confocal microscopy; see chapter 4.2.6). Cytoplasmic processes were clearly visible (when using a higher detector setting; Fig. 5.1c) that became less apparent in the presence of Latrunculin B (5μM; 30mins; 37°C; Fig. 5.1d). The loss of the cell processes is in accordance with the disruption of the actin cytoskeleton as previous work on chondrocytes has shown that actin is a major structural component in cell processes as well as being involved in the regulation of the chondrocytic phenotype (Benya *et al.*, 1981; Benya *et al.*, 1988).

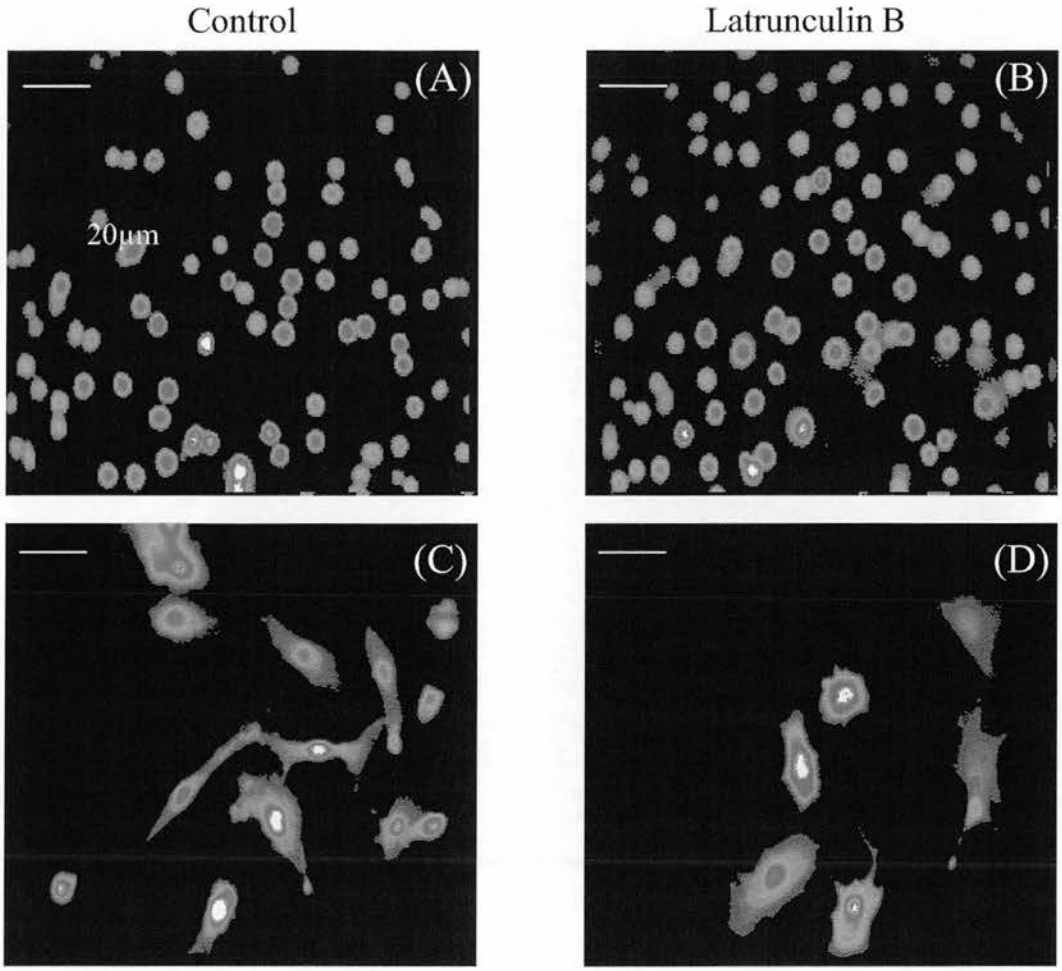


Figure 5.1. Images of fura-2 loaded chondrocytes.

Chondrocytes, freshly-isolated or cultured, were incubated with fura 2 AM (5μM; 30mins; 37°C) or calcein AM respectively (5μM; 30mins; 37°C) and visualised by fluorescent imaging using a PTI Imagemaster™ system. Cells were focused close to the mid-plane though an x40 oil objective and the fura-2 excited alternately at 0.4Hz at 358nm and 380nm. Calcein was excited at 495nm. (A) Image of freshly-isolatedcontrol chondrocytes. (B) Image of freshly-isolated chondrocytes incubated with Latrunculin B (5μM; 30mins; 37°C). (C) Image of 2D cultured control chondrocytes. (D) Image of 2D culture chondrocytes incubated with Latrunculin B (5μM; 30mins; 37°C). Freshly isolated chondrocytes appeared rounded (as viewed through a single optical plane) and this did not seem to be affected by an incubation with Latrunculin B. Conversely, 2D cultured chondrocytes had flattened and had become irregular in shape. After the incubation with Latrunculin B, fewer protrusions were visible and the cells appeared more rounded. These changes in morphology would imply that the Latrunculin B was disrupting the actin. Images are shown as raw, un-altered ***Imagemaster™*** *files. Cells are from 2 joints. Scale bar = 20μm.*

5.2.2 Example of a Latrunculin B Incubated Chondrocyte Volume-Regulating by RVD.

The resting fluorescence of a freshly-isolated fura-2 loaded, Latrunculin B-treated chondrocyte was recorded under constant perfusion with an iso-osmotic 'control saline' (37°C; 380mOsm.kg H_2O^{-1}) for 2mins. The perfusion saline was then switched to deliver a hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) that resulted in a rapid fall in intracellular fluorescence indicating an increase in cell volume. RVD was then recorded for the subsequent 10mins (Fig. 5.2). In this example, the maximal change in fluorescence occurred ~60 seconds post-osmotic challenge with a decrease of 14.7 %. The cell then underwent RVD where the final 358nm fluorescence (measured at 10mins post-osmotic challenge) was not significantly different ($p>0.05$; Student's t-test) to the value recorded in the iso-osmotic 'control' saline.

In response to the hypo-osmotic challenge there was a transient rise in intracellular calcium ($[Ca^{2+}]_i$) that returned to basal levels during the remainder of the experimental period. The maximal rise (23.7 % above basal levels) appeared to correlate with the onset of RVD although it is currently unclear if the change in $[Ca^{2+}]_i$ is implicated in the response.

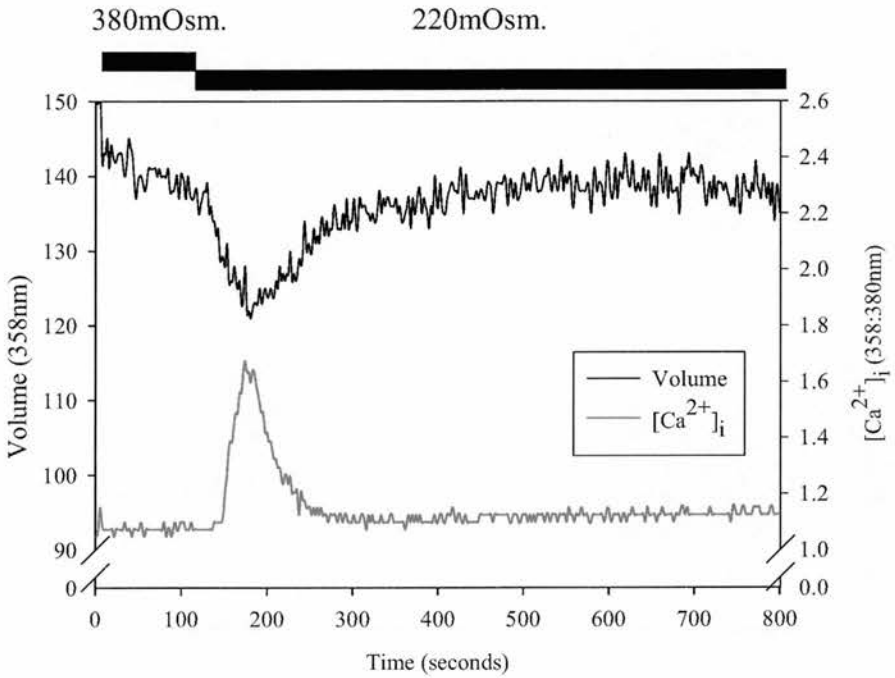


Figure 5.2. Example of a single freshly-isolated, Latrunculin B incubated chondrocyte undergoing RVD.

Chondrocytes were isolated from full depth bovine articular cartilage explants into 380mOsm.kg H_2O^{-1} DMEM and incubated with fura-2 AM (5μM; 30mins; 37°C) and Latrunculin B (5μM; 30mins; 37°C). Cells were perfused with an iso-osmotic saline (380mOsm.kg H_2O^{-1}) and then a 43% hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) was applied. The decrease in osmolality resulted in a fall in the 358nm fluorescence indicating an increase in cell volume. This cell then underwent RVD where at the end of the 10min experimental period there was a complete recovery in cell volume. The decrease in extracellular osmolality resulted in a transient rise in $[Ca^{2+}]_i$ (indicated by an increase in the 358:380nm ratio) that had returned to basal levels at the end of the experimental period. Data are expressed as raw fluorescence trances. N=1 cell.

5.2.3 Effect of 5μM Latrunculin B on Freshly Isolated Chondrocyte RVD.

Having shown the morphology of freshly-isolated with and without the presence of 5μM Latrunculin, the capacity for chondrocyte RVD was then studied by fluorescent microscopy. Cells were incubated with fura-2 AM (5μM; 30mins 37°C) and RVD recorded (as previously described; see Materials and Methods) in response to a 43% hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) delivered by perfusion. To examine the effects of actin de-polymerisation on the capacity for RVD, chondrocytes were incubated with Latrunculin B (5μM; 30mins; 37°C). RVD was then recorded over a 10min experimental period. The Latrunculin was also added to all experimental salines. The subsequent data were then saved and transferred to Excel™ and MAX™ for analysis.

In response to the osmotic challenge, there was a decrease in 358nm fluorescence (as measured by the 510nm emission) indicating an increase in cell volume. There was no significant difference ($p>0.05$; Student's unpaired t-test) in the maximal fluorescent change between control and Latrunculin B treated chondrocytes with a standardised increase in volume of 11.3 ± 0.3 % and 10.7 ± 0.6 % recorded respectively (n=6, N=104).

Data were subsequently plotted and the rate of RVD calculated by linear regression ($r^2>0.9$). There was no significant difference in the rate of RVD (expressed as a t½; time taken for a 50% recovery in volume; see Materials and Methods) between control and Latrunculin B treated chondrocytes. Control chondrocytes performed RVD with a t½ of

6.8 ± 1.17 mins and Latrunculin B treated chondrocytes had t½ of 6.6 ± 0.14 mins (n=6, N=104; Table 5.1).

Despite the fact that there was no significant difference in the rate of RVD, it was possible the Latrunculin B may have altered the number of cells responding. The percentage of chondrocytes showing RVD was calculated as the number of cells showing more than 50% RVD (taken from the point of maximal cell swelling) at the end of the experimental period. There was no significant difference ($p>0.05$; Student's t-test) between control and Latrunculin B treated chondrocytes with 61 ± 6.1 % and 61 ± 11.6 % of cells responding respectively (n=6, N=104). This values are very similar to each other, but a closer inspection of the s.e.m, shows that the response was varied between joints.

As seen in chapter 3, the decrease in extracellular osmolality initiated a transient rise in $[Ca^{2+}]_i$ as shown by an increase in the 358nm:380nm ratio. It was subsequently tested to see if the $[Ca^{2+}]_i$ rise was sensitive to Latrunculin B. In control chondrocytes, 65 ± 8.7 % (taken as a cell showing a rise greater than 20% over basal levels; see Materials and Methods) of cells responded to the hypo-osmotic challenge with a rise in $[Ca^{2+}]_i$. This was not significantly decreased ($p>0.05$; Student's t-test) with an incubation with Latrunculin B where 71 ± 1.6 % of chondrocytes responded (n=6, N=104).

These data show that in response to a 43% hypo-osmotic challenge, bovine articular chondrocytes were able to volume regulate via RVD. The capacity for RVD and the rise in $[Ca^{2+}]_i$ were not inhibited by an incubation with 5μM Latrunculin B.

Measurement	Control	Latrunculin B (5μM)
Maximal fluorescence change (%)	11.3 ± 0.3	10.7 ± 0.6
t½ (mins)	6.8 ± 1.17	6.6 ± 0.14
Cells showing RVD (%)	56 ± 10.0	55 ± 9.6
Cell showing a rise in $[Ca^{2+}]_i$ (%)	65 ± 8.7	71 ± 1.6

Table 5.1. Summary of the *BAC RVD in the presence of 5μM Latrunculin B.*

Chondrocytes were isolated from bovine articular cartilage explants, incubated with fura 2 AM (5μM; 30mins; 37°C) and RVD studied in response to a 43% hypo-osmotic challenge 380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}*). The actin cytoskeleton was disrupted with Latrunculin B (5μM; 30mins; 37°C) and the capacity for RVD compared to control cells. The pre-incubation with the drug had no effect ($p>0.05$; Student's t-test) on the capacity for RVD or the transient rise in $[Ca^{2+}]_i$ associated with a decrease in extracellular osmolality. Data are shown are mean ± s.e.m; n=6 joints, 104 cells.*

5.2.4 The Effect of 5μM Latrunculin B on the Actin Cytoskeleton of Freshly Isolated Chondrocytes.

Data from the previous section (5.2.3) have shown that RVD in freshly-isolated bovine articular chondrocytes was not inhibited by a pre-incubation with 5μM Latrunculin B. This would suggest that the RVD response was not dependent upon a polymerised F-actin cytoskeleton. It is possible that the lack of inhibition by Latrunculin B was due to the actin cytoskeleton not being depolymerised and therefore still able to function in the RVD response. This has been commented on in leukocytes, where both cytochalasin B and D failed to inhibit RVD. It was later found that the actin cytoskeleton remained intact and therefore may still have been involved in the RVD response (Downey *et al* 1995). Here, the depolymerisation of the F-actin cytoskeleton by Latrunculin B was tested in bovine articular chondrocytes by confocal microscopy.

Chondrocytes were isolated from full-depth cartilage explants into 380mOsm.kg H_2O^{-1} DMEM (see Materials and Methods). Cells were then allowed to attach to 12mm coverslips, fixed in 4% paraformaldehyde and the F-actin labelled using Phalloidin-FITC (see Materials and Methods). Images were acquired by CLSM and analysis performed as previously described.

As shown in the confocal z-series (Fig. 5.2A), labelling of actin cytoskeleton with Phalloidin-FITC revealed that the majority of the polymerised F-actin at the chondrocyte periphery (indicated by a 'green ring' of FITC fluorescence at the cell boundary) with very little staining though the cell cytosol. When the z-series was

viewed as a z-stack (sometimes referred to as a "projected image"), the chondrocytes appeared almost completely green. This apparent 'whole cell labelling' was due to all the fluorescent Phalloidin-FITC slices being observed at once and was equivalent to looking down onto the cell from above (Fig. 5.2B). This high level of green intensity implied that the F-actin cytoskeleton was polymerised. When comparing the FITC fluorescence intensity to DNase, there was a higher level of FITC fluorescence therefore implying that more of the cellular actin was in the polymerised state (data not shown).

The incubation with Latrunculin B (5μM; 30mins; 37°C) resulted in the disruption of the actin cytoskeleton as shown by the loss of the peripheral F-actin labelling (Fig 5.3A). Unlike the images acquired in control cells, there was no continuous 'green ring' of FITC fluorescence at the chondrocyte periphery and instead the labelling was punctuate. When visualised as a projected image, there was less FITC fluorescence when compared to control cells (Fig 5.3B). This loss of fluorescence and punctate labelling of the actin would connote that the actin cytoskeleton was disrupted by the presence of the Latrunculin B.

The images (Fig. 5.2B & Fig. 5.3B) were then analysed by linear profiling (see Materials and Methods). In control cells it was found that the maximal FITC fluorescent intensity were towards the edges of the profile (therefore at the cell periphery) with significantly less staining through the cell centre ($p<0.05$). In chondrocytes treated with Latrunculin B, there was a loss of the peripheral actin label and no significant difference ($p>0.05$) in the FITC fluorescent intensity between the cell periphery and the cell cytoplasm (Fig. 5.4A). When comparing the maximal standardised fluorescent

intensities between control and Latrunculin B treated chondrocytes, there was a significant reduction in the peripheral intensity ($p<0.001$; Student's t-test) in the presence of the drug (fig 5.4B; n=32 N=2).

These data show that in freshly-isolated bovine articular chondrocytes, the polymerised actin cytoskeleton was located towards the cell periphery and there was very little polymerised actin running through the cell. A 30min incubation with 5μM Latrunculin B resulted in a disruption of the actin cytoskeleton and therefore in conjunction with the RVD data (see previous), these results suggest that RVD in bovine articular chondrocytes is independent of polymerised F-actin.

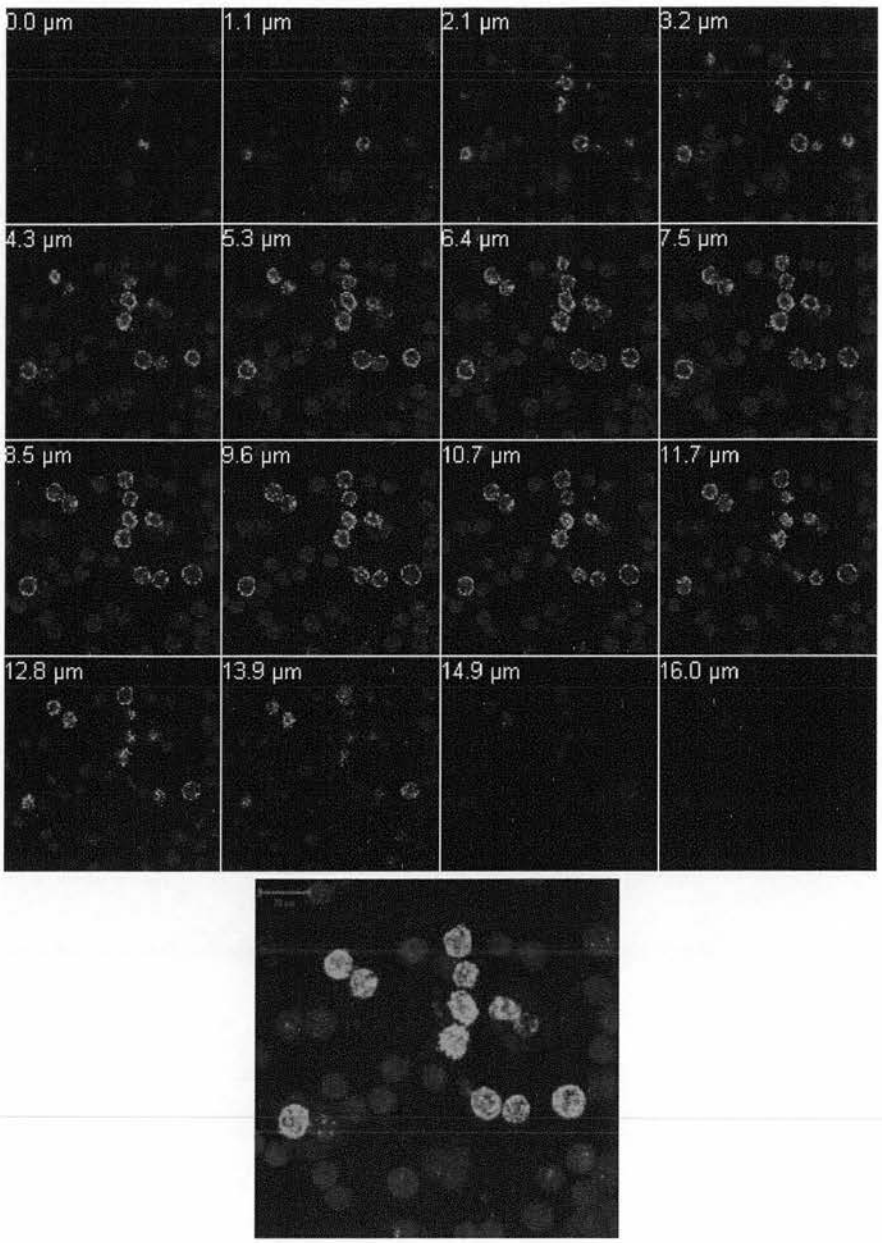
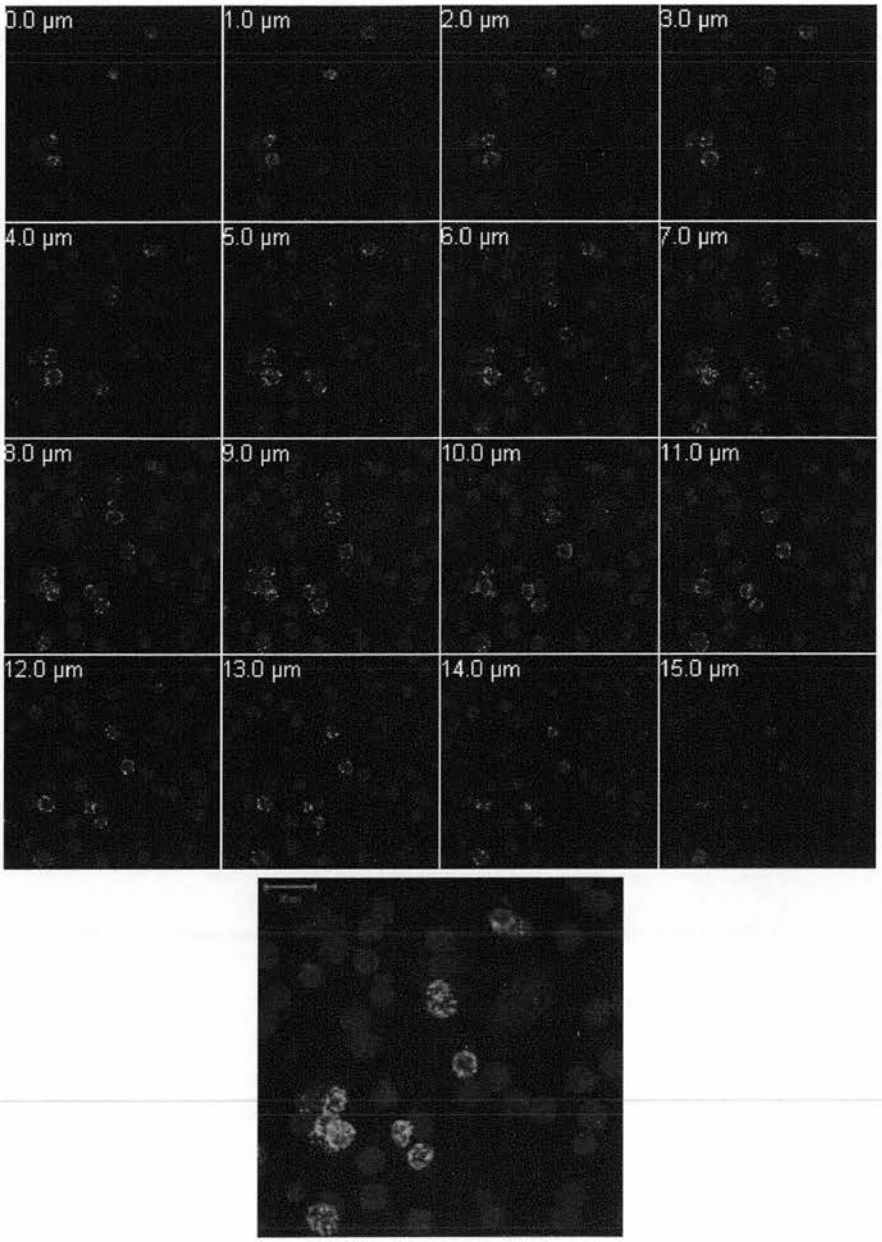


Figure 5.3. Distribution of polymerised actin in freshly-isolated chondrocytes.

Chondrocytes were isolated from cartilage explants, fixed and the polymerised actin cytoskeleton labelled with Phalloidin-FITC. Images were acquired by CLSM (see Materials and Methods) though an x40 oil lens with z-steps of 1.0µm and the power output of the laser adjusted to avoid saturation of the detector. (A), z-series of a region of cells. A region of chondrocytes was selected and the cells imaged. The F-actin was distributed cortically with little labelling across the cell cytosol. (B) The images of the z-stack were then viewed as a projected image (see Materials and Methods) and the chondrocytes appeared almost completely green. This was due to the fact that the FITC from each optical z-slice was being viewed at once and the image is equivalent to an image acquired by epifluorescent microscopy. Cells from one joint.

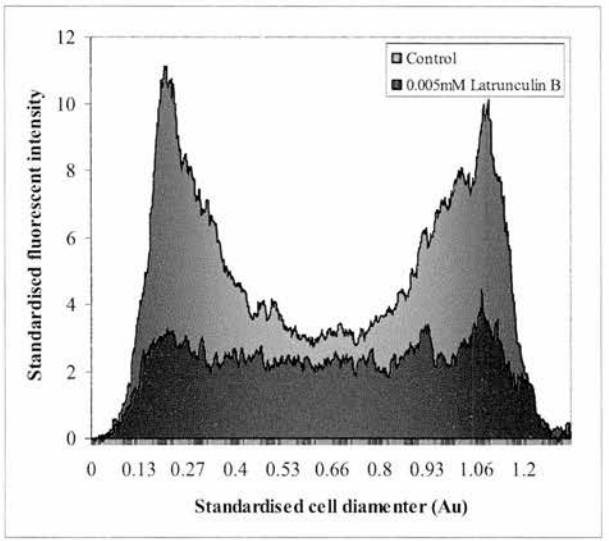


(A)

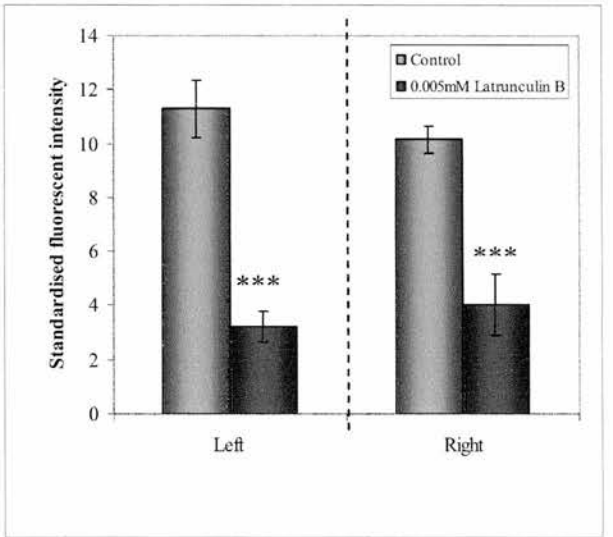
(B)

Figure 5.4. The effects of Latrunculin B on the distribution of polymerised actin in freshly-isolated chondrocytes.

Chondrocytes were isolated, incubated with Latrunculin B (5µM; 30mins; 37°C), fixed and the polymerised actin cytoskeleton labelled with Phalloidin-FITC. (A), z-series of a region of cells. A region of chondrocytes was selected and the cells imaged. There was a loss of the cortical F-actin arrangement (as seen in control cells) and the image was punctuated. (B) The images of the z-stack were then viewed as a projected image (see Materials and Methods) and the chondrocytes appeared less green when compared to control cells. These data would imply that the chondrocyte actin cytoskeleton was broken down by Latrunculin B. Cells from one joint.



(A)



(B)

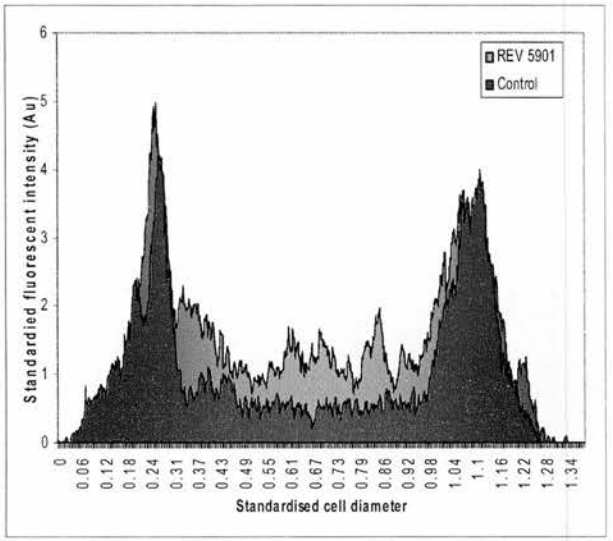
Figure 5. 5. Effects of 5μM Latrunculin B actin on polymerisation in freshly-isolated chondrocytes.

Chondrocytes were isolated from cartilage explants into 380mOsm.kg H_2O^{-1} DMEM, incubated with Latrunculin B (5μM; 30mins; 37°C), fixed and polymerised F-actin labelled with Phalloidin-FITC (RT°C; 40mins). Images were acquired by confocal microscopy and quantified by linear fluorescent analysis (see Materials and Methods). Briefly, linear profiles were drawn through the middle of each cell (through a single z-plane; perpendicular in the x-y plane) and the fluorescent intensity calculated using Zeiss imaging software. For each cell, the profiles were averaged and standardised to the cell diameter. (A) Area plot of the FITC intensity. The FITC intensity was plotted for control (blue) and Latrunculin B (red) treated chondrocytes against the standardised cell diameter. In control chondrocytes, the maximal intensity was towards the cell periphery indicating that the actin was cortically distributed. (B) The maximal cortical FITC fluorescence was subsequently plotted for control and Latrunculin B treated chondrocytes and plotted as fluorescence at the cell edge; termed 'left' and 'right'. There was a significant ($p<0.001$; Student's t-test) reduction in the cortical actin intensity indicating that the Latrunculin B disrupted polymerised actin; ($n=32$, $N=2$).

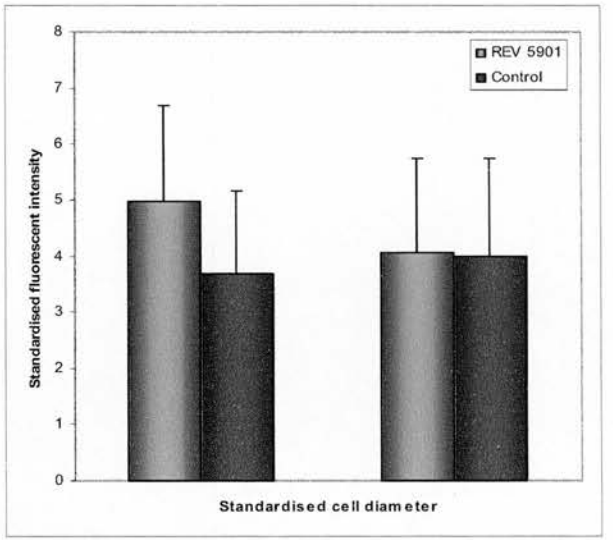
5.2.5 The Effect of REV 5901 on the Actin Cytoskeleton of Freshly Isolated Chondrocytes.

It has previously been shown that an incubation with REV 5901 (50μM; 30mins; 37°C) inhibited RVD in freshly-isolated bovine articular chondrocytes (Hall & Kerr, 2000; Kerrigan & Hall, 2000). Despite there being no inhibition of RVD due to the depolymerisation of the actin cytoskeleton with Latrunculin B, the effects of REV 5901 were tested on the actin cytoskeleton. Chondrocytes were isolated and plated onto 12mm sterile (autoclaved) coverslips in 380mOsm.kg H_2O^{-1} DMEM. Cells were subsequently incubated with REV 5901 (as above) and labelled as previously described (See Materials and Methods). Images were then acquired by CLSM and analysis performed using linear profiling.

Preliminary data has shown that an incubation with REV 5901 had no significant effect ($p>0.05$) on the actin cytoskeleton of freshly-isolated chondrocytes. When visualised by CLSM, the cortical arrangement was still present as indicated by the 'green ring' of FITC fluorescence located at the cell boundary. Using linear profiling, no significant difference ($p>0.05$; Student's t-test) was found in the maximal cortical FITC fluorescence between control and REV 5901 treated chondrocytes (Fig. 5.6a & Fig 5.6b; n =2, N=17). This would imply that the polymerised actin cytoskeleton is not affected by this treatment.



(A)



(B)

Figure 5. 6. Effects of 50μM REV 5901 actin polymerisation in freshly-isolated chondrocytes

Chondrocytes were isolated from cartilage explants into 380mOsm.kg H_2O^{-1} DMEM, incubated with REV 5901 (5μM; 30mins; 37°C), fixed and polymerised F-actin labelled with Phalloidin-FITC (RT°C; 40mins). Images were acquired by confocal microscopy and quantified by linear regression analysis (see Materials and Methods). Briefly, linear profiles were drawn through the middle of each cell (through a single z-plane; perpendicular in the x-y plane) and the fluorescent intensity calculated using Zeiss imaging software. For each cell, the profiles were averaged and standardised to the cell diameter. (A) Area plot of the FITC intensity. The FITC intensity was plotted for control (green) and REV 5901 (blue) treated chondrocytes against the standardised cell diameter. In both control chondrocytes and REV 5901 treated chondrocytes, the maximal fluorescent intensity were towards the cell periphery indicating that polymerised actin was cortically distributed. (B) The maximal cortical FITC fluorescence was subsequently plotted for control and REV 5901 treated chondrocytes and plotted as fluorescence at the cell edge; termed 'left' and 'right'. There was no significant ($p>0.05$; Student's t-test; t-test performed between cells and not joints due to N=2 joints) difference and therefore would imply that REV 5901 does not cause depolymerisation of the actin cytoskeleton; (n=17, N=2).

5.2.6 The Effect of Latrunculin B on RVD in 2D Cultured Chondrocytes

During time in 2D culture, chondrocytes de-differentiate into the fibroblastic phenotype resulting in marked changes in the products of metabolism, the structure of the actin cytoskeleton and the capacity for RVI (Benya & Shaffer, 1981; Benya *et al.*, 1981; Benya & Shaffer, 1982; Benya *et al.*, 1988; section 4.2.7). In freshly-isolated chondrocyte the polymerised actin cytoskeleton is located cortically with little spanning of the cell cytoplasm (Guilak *et al.*, 2002; Erickson *et al.*, 2003). After time in 2D culture, there was drastic re-modelling resulting in the formation of cell-length stress fibres and the loss of the distinctive differentiated cortical arrangement (Langelier *et al.*, 2000).

As time in 2D culture results in a change in the organisation of the F-actin cytoskeleton, it is possible that there may also be a change in the RVD response. Therefore, the capacity for RVD was studied in 2D cultured chondrocytes in response to a 43% hypo-osmotic challenge in the presence of Latrunculin B (5μM; 30mins; 37°C). Cells were cultured in 2D for two weeks (see Materials and Methods) incubated with fura-2 AM (5μM; 30mins 37°C) and RVD recorded by fluorescent microscopy.

There was no significant difference ($p>0.05$; Student's unpaired t-test) in overall maximal volume swelling between control and Latrunculin B treated chondrocytes. In control cells there was a maximal fluorescence change of 16.04 ± 1.83 % compared to 18.0 ± 0.178 % recorded in Latrunculin B treated chondrocytes (n=3 joints, 117 cells).

Data were then plotted and the rate of RVD calculated by linear regression and extrapolation (shown as a t½; see Material and Methods). The percentage of cells responding was also calculated. A cell was deemed to have shown RVD if at 10mins post-osmotic challenge fluorescence had recovered by at least a 50 % from the maximal rise. In control chondrocytes, 35.3 ± 5.3 % of cells underwent RVD with a t½ of 8.25 ± 1.75mins. This was not significantly altered by the pre-incubation with Latrunculin B were 42.9 ± 2.97% responded with a t½ of 10.0 ± 0.5mins. (n=3 joints, 117 cells; Table 5.2).

These data show that despite the re-modelling of the actin cytoskeleton during time in 2D culture, RVD was still independent of actin polymerisation. Latrunculin B did not alter the maximal cell swelling, the percentage of cells responding or the efficacy of the response. This would therefore suggest that despite the chondrocyte actin cytoskeleton being depolymerised by Latrunculin B, RVD remained unaffected and therefore not dependent upon actin polymerisation. This may suggest that the method of volume regulation between 2D and freshly-isolated chondrocytes is mediated by the same pathway and does not involve the actin cytoskeleton.

Measurement	Control	5µM Latrunculin B
Maximal fluorescence change (%)	16.04 ± 1.83	18.0 ± 0.178
t½ (mins)	8.25 ± 1.75	10.0 ± 0.5
Cells showing RVD (%)	35.3 ± 5.3	42.9 ± 2.97

Table 5.2. Summary of the cBAC RVD in the presence of 5µM Latrunculin B.

Chondrocytes were isolated from bovine articular cartilage explants into 380mOsm.kg H_2O^{-1} DMEM and cultured in 2D for 3 weeks (see Materials and Methods). Prior to experimentation, cells were passaged onto 22mm coverslips and incubated with calcein AM (5µM; 30mins; 37°C) and then RVD studied in response to a 43% hypo-osmotic challenge 380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) by perfusion. The actin cytoskeleton was disrupted with Latrunculin B (5µM; 30mins; 37°C) and the capacity for RVD compared to control cells. The pre-incubation with the drug had no affect ($p>0.05$) on the capacity for RVD. Data shown are mean ± s.e.m; n=3 joints, 117 cells.

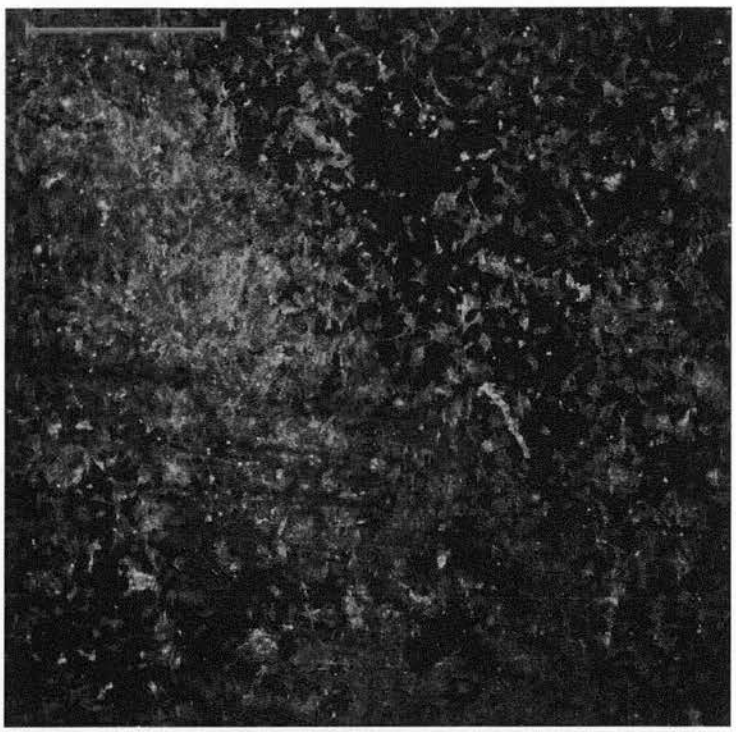
5.2.7 The Effect of Latrunculin B on the Actin Cytoskeleton of 2D Cultured Chondrocytes.

The work presented previously (section 5.2.6) has shown that the RVD response was insensitive to a 30min incubation with 5μM Latrunculin B. In this section, the affect of Latrunculin B on actin polymerisation was studied to ensure that there was actin depolymerisation as a result of incubation with the drug. Chondrocytes were cultured for 3 weeks and passaged onto 12mm coverslips (see Materials and Methods). The polymerised (F) actin was labelled with Phalloidin-FITC (25μl/ml) and the monomeric (G) actin labelled with DNase-Texas Red (2μl/ml). To examine the affects of Latrunculin B (5μM; 30mins; 37°C), the chondrocytes were incubated with the drug for 30mins prior to fixation. Images were then acquired by CLSM (see Materials and Methods).

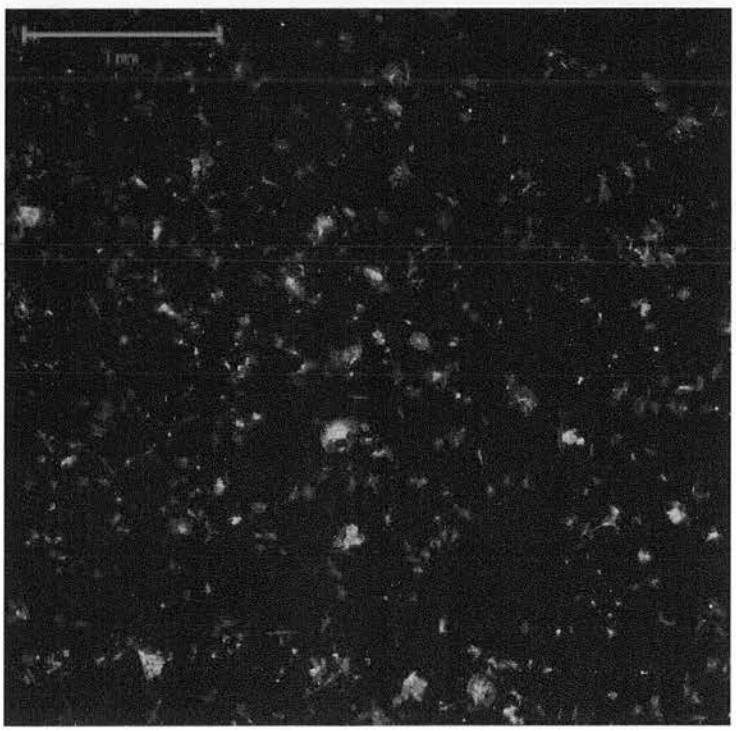
Using 'tile scanning' a large number of cells were visualised. After three weeks in culture the chondrocytes had flattened and appeared to have reverted to a more fibroblastic phenotype. The polymerised (F) actin was no longer restricted to the cell periphery and instead stress fibres were clearly visible running throughout the cell (Fig. 5.7a). Conversely, after a 30 minute pre-incubation with Latrunculin B (5μM; 37°C) the amount of polymerised actin appeared to decrease and less stress fibres were visible (Fig 5.7b). There was an increase in the DNase-Texas Red fluorescence that would imply there was an increase in the amount of monomeric (G) actin in the cells.

To examine the structure of the actin cytoskeleton in more detail, images were acquired by CLSM though an x40 objective (Fig. 5.8a & Fig. 5.8b). In control chondrocytes the polymerised actin stress fibres were clearly visible running across the cell. Conversely, a pre-incubation with Latrunculin B resulted in a loss of the polymerised F-actin structure and instead the image appeared more punctate. There was a decrease in the FITC fluorescence and an increase in the DNase-Texas Red fluorescence which is consistent with a switch from polymerised (F) actin to monomeric (G) actin. These changes are further marked in images acquired by electronically zoomed (x4) CLSM (Fig. 5.8c & Fig. 5.8d). As previously described, a 30min pre-incubation with 5μM Latrunculin B resulted in a loss of the organised polymerised (F) actin structure and an increase in monomeric (G) actin.

These data show that after 3 weeks in 2D culture the actin cytoskeleton of bovine articular is re-modelled resulting in the formation of stress fibres and the loss of the cortical structure. A pre-incubation with Latrunculin B, caused the F-actin to depolymerise. Therefore, in conjunction with the previous RVD data, RVD in 2D culture bovine articular chondrocytes is independent of the polymerised actin cytoskeleton.



(A)



(B)

Figure 5.7. The Effects of Latrunculin B actin polymerisation in 2D cultured chondrocytes.

Chondrocytes were isolated from full depth bovine articular cartilage and cultured in 2D for 3 weeks (see Materials and Methods). Prior to experimentation, cells were passaged and the actin cytoskeleton and labelled with Phalloidin-FITC and DNase-Texas Red for polymerised (F) and monomeric (G) actin respectively. Images were acquired using a Zeiss Axiovert LSM510 confocal microscope by tile scanning through an x10 air objective. (A) Control cells. After three weeks in 2D cultured the chondrocytes had flattened and resembled the fibroblastic phenotype. The cortical polymerised actin arrangement as seen in freshly-isolated chondrocytes was no loner apparent and instead stress fibres were running though the cells. In places the image appears out of focus and this is due to the fact that when tile scanning only a single z-plane can be used. (B) Latrunculin B treated cells. After a pre-incubation with Latrunculin B (5μM; 30mins; 37°C), there appeared to be a loss of polymerised actin. Stress fibres were no longer apparent and there was a loss of the green FITC fluorescence. Conversely, there appeared to be an increase in the DNase-Texas Red fluorescence. These data would imply that Latrunculin B disrupted the actin cytoskeleton resulting in a switch from polymerised (F) actin to monomeric (G) actin. Data are shown as un-modified confocal images.

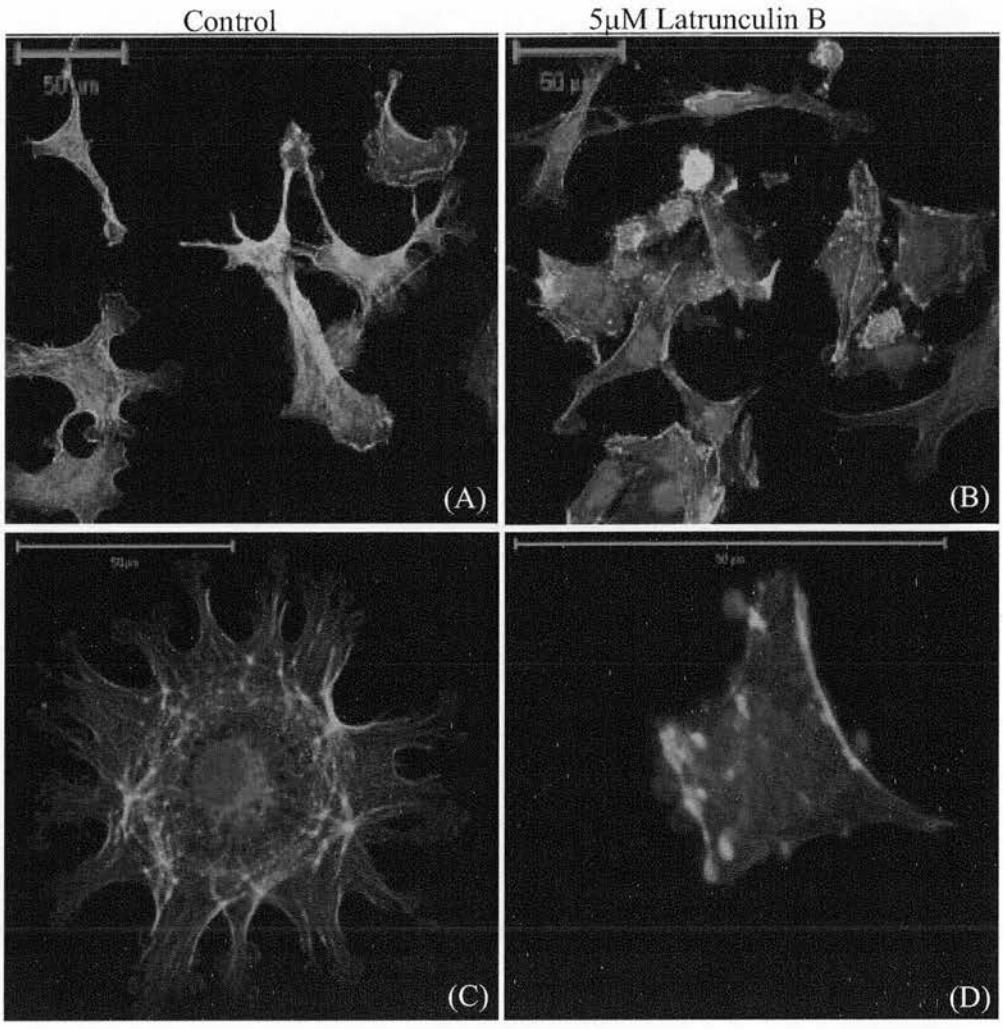


Figure 5.8. Projected images of 2D cultured chondrocytes.

Chondrocytes were isolated from full depth bovine articular cartilage and cultured in 2D for 2 weeks (see Materials and Methods). Prior to experimentation, cells were passaged and the polymerised (F) and monomeric (G) actin respectively. Images were acquired using a Zeiss Axiovert LSM510 confocal microscope. (A) Control chondrocytes imaged using an x40 oil objective. As a result of 2D culture, there had been re-modelling of the actin cytoskeleton and stress fibres are clearly visible. (B) Latrunculin B treated chondrocytes imaged using an x40 oil objective. As a result of the Latrunculin B, the actin cytoskeleton had started to depolymerise as shown by a decrease in the FITC fluorescence and an increase in the DNase-Texas red fluorescence. (C) Control chondrocytes imaged using an x40 oil objective plus x4 zoom. The stress fibres of the chondrocytes are clearly visible. (D) Latrunculin B treated chondrocytes imaged using an x40 oil objective plus x4 zoom. The FITC fluorescence had decreased when compared to control cells and became punctate. Data are un-modified confocal images, n=2 joints.

5.3.0 Results Summary.

The data presented in this chapter has shown that both freshly-isolated and 2D cultured chondrocytes were able to volume regulate via RVD in response to a decrease in extracellular osmolality. A pre-incubation with the actin 'monomeric capping' drug, Latrunculin B had no effect on the capacity for RVD despite it having been shown to break down the polymerised F-actin cytoskeleton. When comparing the capacity for RVD, and the effects of Latrunculin B between freshly-isolated and 2D cultured chondrocytes, there was no significant difference despite a considerable re-organisation of the F-actin cytoskeleton. The rise in $[Ca^{2+}]_i$, observed in freshly-isolated chondrocytes in response to a decrease in extracellular osmolality was insensitive to actin depolymerisation.

5.4.0 Chapter Discussion.

The data presented in this chapter have shown that RVD in response to a decrease in extracellular osmolality was not inhibited by the depolymerisation of the F-actin cytoskeleton by Latrunculin B. Furthermore, in 2D cultured chondrocytes, there was no difference in the dependence on the F-actin cytoskeleton despite significant remodelling during culture. The decrease in extracellular osmolality initiated a transient rise in $[Ca^{2+}]_i$ in some cells, that was not inhibited by F-actin depolymerisation and subsequently returned to basal levels during the remainder of the experimental period.

The actin cytoskeleton has been shown to be an important mediator in both the regulation of the chondrocytic phenotype and signal transduction in response to membrane stretch (Benya & Shaffer, 1981; Wright *et al.*, 1996; Wright & Salter, 1996; Millward-Sadler *et al.*, 1999). In other cell types, it has also been shown that an effective RVD response is dependent upon the polymerised actin cytoskeleton either by direct action on the channel (Reynier *et al.*, 1991; Matthews *et al.*, 1998) or as part of the 'mediator complex' (Cornet *et al.,* 1993; Pedersen *et al.,* 1999; Hoffmann, 2000; Pedersen, *et al.*, 2001). Therefore, the aim of this study was to determine the dependence of RVD upon the polymerised F-actin cytoskeleton. Furthermore, as time in 2D culture influenced the chondrocytes, response to changes in extracellular osmolality (see sections 3.2.6 and 4.2.7) comparisons were performed between freshly-isolated and 2D cultured chondrocytes.

In response to the decrease in extracellular osmolality, both freshly-isolated and 2D cultured chondrocytes underwent RVD with equal efficacy, uninhibited in the presence

of the actin 'monomeric capping agent', Latrunculin B (Tables 5.1 & 5.2). This would seem to imply that F-actin polymerisation (or further organisation as a result of culture), or indeed de-polymerisation in response to the change in osmolality is not required to mediate the response. It is important to note that this study employed both real time fluorescent analysis of RVD and confocal structural analysis of the actin cytoskeleton. It could be suggested that the addition of a drug known to depolymerise the actin cytoskeleton is not enough to prove a lack of involvement of the actin cytoskeleton (Pedersen *et al.,* 1999; Hoffmann, 2000) and therefore, both need to be studied to fully understand the role of the actin cytoskeleton in a cellular responses. In HL-60 cells and human peripheral blood neutrophils, both cytochalasin B and cytochalasin D failed to inhibit the RVD response (Downey *et al.*, 1992; Downey *et al.*, 1995). When tested, it was found that there had been no significant depolymerisation of the actin cytoskeleton and there the role of the actin cytoskeleton remained unclear. This would suggest that different cell types have different sensitivities to actin depolymerising agents, and therefore the affects needs to be studied in conjunction with the rest of the experiment. In this study, the confocal experiments were able to show actin depolymerisation as a result of Latrunculin B treatment. Therefore, the fact that there was no inhibition of the RVD response would suggest that RVD was not dependent upon the polymerised F-actin cytoskeleton.

In the present study, the F-actin cytoskeleton was depolymerised using Latrunculin B. It is a potent disruptor of the actin cytoskeleton and prevents any re-polymerisation that may occur (Wakatsuki *et al.*, 2001). Unlike the cytochalasins, Latrunculin B as well as being a more potent inhibitor of actin depolymerisation is not known to have adverse

affects on other cellular functions (Wakatsuki *et al.*, 2001). Conversely, as shown in Table 1.3, many of the cytochalasins have adverse side-effects, for example, cytochalasin B has been shown to inhibit glucose transport and cytochalasin D inhibits low conductance K^+ channels (Aszalos *et al.*, 1994; Tanaka *et al.*, 1994; Theodoropoulos *et al.*, 1994; Wang *et al.*, 1994; Sasaki *et al.*, 1995). These secondary affects may influence the interpretation of experimental data. This comment is further illustrated by observations made on RVD studies performed in EAT cells where a pre-incubation with both cytochalasin B and cytochalasin D used at depolarising concentrations only cytochalasin B inhibited the RVD response (Pedersen *et al.*, 2001). Interestingly, cytochalasin D has been shown to be more specific at depolymerising F-actin (Aszalos *et al.*, 1994; Wang *et al.*, 1994; Sasaki *et al.*, 1995) and therefore the observed effect may not be a result of F-actin depolymerisation and may relate to one of the 'side effects'. As Latrunculin B is not known to have any adverse cellular functions, the lack of inhibition of RVD can be directly related to the action of the drug and not by the disruption of another cellular pathway.

It has been shown that chondrocyte actin depolymerisation is mediated by a rise in $[Ca^{2+}]_i$, the subsequent activation of gelsolin (an actin cytoskeletal regulatory protein involved in the depolymerisation and subsequent capping of G-actin) and a decrease in the cellular PIP_2 pool (due to the conversion of PIP_2 to IP_3 and DAG; (Erickson *et al.*, 2003). PIP_2 mediates the reversal of the gelsolin effect and therefore an increase in cellular IP_3 (and the consequential decrease in PIP_2) has been suggested as optimal conditions for actin depolymerisation in chondrocytes. The time course for the gelsolin-mediated actin depolymerisation (Erickson *et al.*, 2003) and RVD shown in this chapter

would imply that, the onset of RVD (measured at 37°C) is before the maximal depolymerisation of the actin cytoskeleton although after the co-localisation of gelsolin with actin. RVD is then complete (in most cells) before the re-polymerisation of the actin cytoskeleton (based on the data on porcine articular chondrocytes by Erickson *et al.*, 2003). This may then imply that the polymerised F-actin cytoskeleton could still mediate the initiation of the RVD response although once activated the process of RVD is not dependent upon polymerisation.

When taking into account the incubation period and the slight experimental delay, it would appear that the polymerised F-actin cytoskeleton was not a mediator in the chondrocyte RVD response. Latrunculin B was added to the experimental coverslips 30mins prior to an experiment and this added to the 5mins for the experimental set-up would constitute 35min incubation prior to the osmotic challenge. Therefore, during this period the F-actin cytoskeleton would have depolymerised prior to the onset of RVD. It would further imply that the process of actin depolymerisation is not required to activate the chondrocyte 'osmolyte channel'. RVD independent of F-actin depolymerisation has been shown in B-lymphocytes where the activation of the volume-regulated Cl^- current under iso-osmotic conditions was not stimulated by the addition of cytochalasin B (Levitan *et al.*, 1995). Furthermore, once initiated, the RVD response was not inhibited by actin depolymerisation, although interestingly there was an up-regulation of the Cl^- current (VSAC) upon cytochalasin treatment at a higher osmotic challenge (Levitan *et al.*, 1995). It is therefore possible that the activation of the 'osmolyte channel' is similar to that of the B-lymphocyte VSAC with respect to the F-actin cytoskeleton (i.e. not

dependent upon F-actin depolymerisation) although the effects on F-actin depolymerisation and the activation of the 'osmolyte channel' still remain to be tested.

As mentioned previously, a change in osmolality may be perceived as a mechanical stimulus; (Low & Taylor, 1998). In cultured human articular chondrocytes, pressure-induced strain, results in the influx of calcium and the subsequent activation of SK channels via an $\alpha 5\beta 1$ integrin receptor-ligand complex (Wright *et al.*, 1996; Millward-Sadler *et al.*, 1999). The response can be inhibited by F-actin depolymerisation mediated by cytochalasin D (Wright & Salter, 1996). The data presented in this chapter would suggest that RVD in both cultured and freshly-isolated chondrocytes is not mediated via this pathway, as Latrunculin B did not inhibit the RVD response. This would therefore imply that the mechanism for responding to membrane stretch might be different to 'sensing' or responding to changes in extracellular osmolality. There is also the possibility that that freshly-isolated chondrocytes do not have a functional $\alpha 5\beta 1$ integrin receptor-ligand complex. Cell passage results in the rounding of cell and the detachment from the culture surface. Cell rounding most likey caused a de-polymerisation of the F-actin cytoskeleton (personal observations) and therefore this would lead to in the inactivation of the $\alpha 5\beta 1$ integrin receptor-ligand complex. Furthermore, RVD has been shown in freshly-passaged bovine articular chondrocytes following a ten day culture and a trypsin passage (Yellowley *et al.*, 2002). These observations in conjunction with the data present in this chapter would suggest that the $\alpha 5\beta 1$ integrin complex is not involved in bovine articular chondrocyte RVD.

In bovine articular chondrocytes, RVD is mediated principally by a non-specific 'osmolyte channel' resulting in the efflux of osmolytes e.g. K^+, Cl^- and taurine (Hall, 1995; Bush & Hall, 2000, 2001b, a; Hall & Bush, 2001). Currently the mechanism in which the RVD response is activated in not clear, although it is thought that activation is not mediated by components of the arachidonic cascade (Hall & Kerr, 2000). As the pre-incubation with Latrunculin B did not (attenuate or) inhibit the RVD response, the data presented in here may support the fact the channel regulation is not mediated by translocation of transporter from a cellular pool, as the process of transporter exocytosis requires an intact actin cytoskeleton (Hoffmann & Mills, 1999; Pedersen *et al.*, 2001). This observation is further supported by measurements made on the ouabain- and bumetanide-insensitive, volume activated K^+ efflux recorded in isolated bovine articular chondrocytes (Hall *et al.*, 1996b). It was shown that the activation of the efflux was rapid (under 20seconds) suggesting the channel was already present in the plasma membrane. Furthermore, it may also imply that the 'osmolyte channel' is not directly regulated by the polymerised F-actin cytoskeleton as previously observed for both the NKCC and the NHE transport systems (see section 1.9.4; Matthews *et al.*, 1994; Matthews *et al.*, 1997; Szaszi *et al.*, 2000).

Another potential regulator of the chondrocyte 'osmolyte channel' could be intracellular calcium. In chondrocytes, a decrease in extracellular osmolality has been shown to initiate a Gd^{3+} sensitive $[Ca^{2+}]_i$, transient rise in mediated by an influx of calcium across the plasma membrane and a release from of calcium IP_3 sensitive calcium stores (Yellowley *et al.*, 2002; Erickson *et al.*, 2003). Latrunculin B failed to inhibit the $[Ca^{2+}]_i$ rise in this study and therefore it would appear the rise is not dependent upon a

polymerised F-actin cytoskeleton. The lack of inhibition of the $[Ca^{2+}]_i$ transient by actin depolymerisation (using cytochalasin D) has also been observed in porcine intervertebral disk chondrocytes in response to an increase in extracellular osmolality (Pritchard *et al.*, 2002) although interestingly, the response was inhibited by the stabilisation of the actin cytoskeleton by phalloidin. Whether the rise in $[Ca^{2+}]_i$ as a result of a hyper-osmotic challenge is mediated by the same mechanism as a hypo-osmotic challenge has yet to be proven, although both are due to a calcium influx resulting in an $[Ca^{2+}]_i$ transient lasting for the same length of time with the same magnitude (Kerrigan & Hall; unpublished observations).

It is possible that the observed changes in $[Ca^{2+}]_i$ are not involved in the onset of RVD and may just be involved in the regulating the organisation of the actin cytoskeleton (Erickson *et al.*, 2003). *In vivo*, chondrocytes are under constant mechanical stress with constant changes in extracellular osmolality as a result of joint articulation (Urban, 1994; Wilkins *et al.*, 2000a). It is therefore conceivable that there will be concomitant changes in the organisation of the F-actin cytoskeleton as it is involved in maintaining the viscoelastic properties of the chondrocyte (Guilak *et al.*, 2000; Pritchard *et al.*, 2002). This in conjunction with the fact that F-actin depolymerisation (and changes in osmolality) have been shown to a decrease in the elastic and viscoelastic properties of chondrocytes (Trujillo *et al.*, 1999), could imply the importance of the F-actin cytoskeleton in maintaining cell viability, resistance to mechanical damage and thus preventing the degenerative state.

In chondrocytes, the regulation of the F-actin cytoskeleton involves changes in $[Ca^{2+}]_i$ and regulation by a gelsolin mediated pathway (Erickson *et al.*, 2003). Based on the data presented in this chapter, it has already been suggested that as the actin cytoskeleton was already depolymerised prior to the addition of the hypo-osmotic media and therefore the rise in $[Ca^{2+}]_i$ may not be involved in the regulation of the F-actin cytoskeleton. If this were the case, the most likely explanation for the $[Ca^{2+}]_i$ rise is in mediating the RVD response and in chapter three, it was suggested that some chondrocytes have a dependence on intracellular calcium for volume regulation. Alternatively, it is possible that chondrocytes from the three main zones of cartilage have a different resilience to Latrunculin B induced F-actin depolymerisation as observed in human leukocytes; (Downey *et al.*, 1992; Downey *et al.*, 1995). Chondrocytes from the SZ have a more dense cortical F-actin cytoskeleton when compared to the MZ and DZ chondrocytes (thought to provide mechanical resilience; (Langelier *et al.*, 2000) and it is possible that 5μM Latrunculin B may not have fully depolymerised the F-actin cytoskeleton. If this were the case, and the F-actin cytoskeleton was not depolymerised by the Latrunculin B, then it is possible that the rise in $[Ca^{2+}]_i$ could be involved in the maintenance of the F-actin cytoskeleton by one population of chondrocytes (potentially from the SZ) and RVD in another population of chondrocytes (potentially MZ and DZ). To resolve this, *in situ* confocal studies need to be performed to fully elucidated the roles of $[Ca^{2+}]_i$ and F-actin in RVD in relation to changes in extracellular osmolality.

In summary, RVD in bovine articular chondrocytes is mediated principally via an 'osmolyte channel' (Bush & Hall, 2000, 2001b) whose activation is insensitive to the

depolymerisation of the F-actin cytoskeleton. The rise in $[Ca^{2+}]_i$ (as a result of the decrease in extracellular osmolality) did not appear to correlate with the capacity for RVD and was also found not to be inhibited by the depolymerisation of the F-actin cytoskeleton. Time in 2D culture, unlike chondrocyte RVI, had no affect on the RVD response when compared to freshly-isolated cells despite a significant re-organisation of the cytoskeleton including the formation of actin stress cables. It is still unclear exactly how the 'osmolyte channel' is regulated and the exact role of the $[Ca^{2+}]_i$ rise remains to be elucidated.

Comparison of RVD in Chondrocytes Isolated from 'Degenerate' and 'Non-Degenerate' Human Articular Cartilage

6.1.0 Chapter Introduction

Articular cartilage protects the underlying bone from the compressive and shearing forces generated from joint articulation (Weiss *et al.*, 1968). Chondrocytes, the only resident cell type produce, maintain and adapt the cartilage matrix to the physico-chemical environment and thus maintaining the mechanical resilience of the tissue (Stockwell, 1979; Stockwell, 1991). Cartilage principally consists of collagen (~15-25%) and proteoglycan (~3-10%) and it is these two components and how they influence cartilage hydration that endow the cartilage with its protective properties (Maroudas, 1980). The organisation of the collagen (principally type II) is such that the negatively charged PGs are held below their fully hydrated volume (due to the Donnan equilibrium, the unusual ionic nature of the cartilage matrix and the resistance from the collagen fibrils) thus establishing a swelling force within the cartilage matrix (Urban, 1994). Therefore, a loss of organisation of the collagen fibrils or changes in PG content will have a significant effect on the mechanical properties of the cartilage.

In osteoarthritis (OA), the cartilage is significantly overhydrated when compared to non-osteoarthritic cartilage as a result of damage to the collagen network (Grushko *et al.*, 1989; Stockwell, 1991; Bank *et al.*, 2000). Normally, collagen fibrils within cartilage are highly organised and crossed linked principally by collagen type IX and type XI (Eyre & Wu, 1995) forming a resilient network. Damage to either the collagen type II fibrils or the cross-linking collagens, decreases the network integrity and results in the further swelling of the cartilage PG. Recently it has been suggested that it is the damage to the collagen fibrils (by cleavage) and not the crosslinks that result in cartilage

overhydration (Bank *et al.*, 2000). The increase in cartilage hydration has three significant effects on matrix biology. The first is the loss of the load-bearing properties of cartilage (Stockwell, 1991). The second, as PG confer the relative pore-size and permeability of matrix the rate of fluid loss would be increased (Maroudas, 1980); (Grushko *et al.*, 1989). Thirdly, the extracellular osmolality of the tissue will be decreased. This final effect will now be focused upon.

An increase in cartilage hydration will have two effects on chondrocytes. The first will result in an increase in cell volume, and recently in human articular chondrocytes it has been shown that volume increases with cartilage degeneration (Bush & Hall, 2003). Secondly, a reduction in extracellular osmolality will most likely decrease matrix synthesis (Urban *et al.*, 1993) potentially exacerbating the degeneration process. Interestingly, it has also been shown that volume measured in chondrocytes from OA cartilage is volume greater than that predicted by free-swelling due to cartilage hydration (Bush & Hall, 2003). It is therefore possible that chondrocytes from degenerate cartilage have an impaired RVD response and will continue to swell and as the hydration of the cartilage increases.

Using fluorescent imaging of chondrocytes isolated from 'non-degenerate' and 'degenerate' cartilage (see section 2.6.0) the capacity for RVD was measured in response to a 43% hypo-osmotic challenge. Furthermore, as human articular chondrocytes have been shown to respond to mechanical stimulation via an $\alpha 5\beta 1$ integrin receptor-ligand complex, and it was conceivable that this could be involved in the transduction of changes in cell volume the effects of Gd^{3+} (an inhibitor of stretch-

sensitive channels and of the α5β1 integrin response) was also tested. Furthermore, in bovine articular chondrocytes it has been shown that RVD could be inhibited by REV 5901 and therefore the effects of this drug on isolated human articular chondrocyte RVD was studied. Finally, as changes in $[Ca^{2+}]_i$ have been implicated in the RVD in some cell types, changes in $[Ca^{2+}]_i$ were correlated to the capacity for RVD.

Hypothesis to be tested:

Human articular chondrocyte RVD involves the α5β1 integrin receptor-ligand complex and chondrocytes isolated from 'degenerate' cartilage have an impaired RVD response.

6.2.0 Results

6.2.1 Fluorescent Images of Isolated Human Articular Chondrocytes

Fura-2 loaded chondrocytes isolated from human articular cartilage were visualised by fluorescent microscopy following isolation into 380mOsm.kg H_2O^{-1} DMEM (see Materials and Methods). When viewed by fluorescent microscopy, the fura-2 appeared to be distributed evenly throughout the chondrocytes with no apparent areas of punctate fluorescence.

The chondrocytes appeared spherical, with a variation in apparent cell size. Some of the cells were not completely isolated (Fig. 6.1). Instead, pairs were often observed and in some experiments, clumps were clearly visible. This could be an indication from where the chondrocytes resided *in situ*; the smaller chondrocytes possibly originating from the surface zone (SZ) and the larger chondrocytes from the mid and deep zones (MZ & DZ).

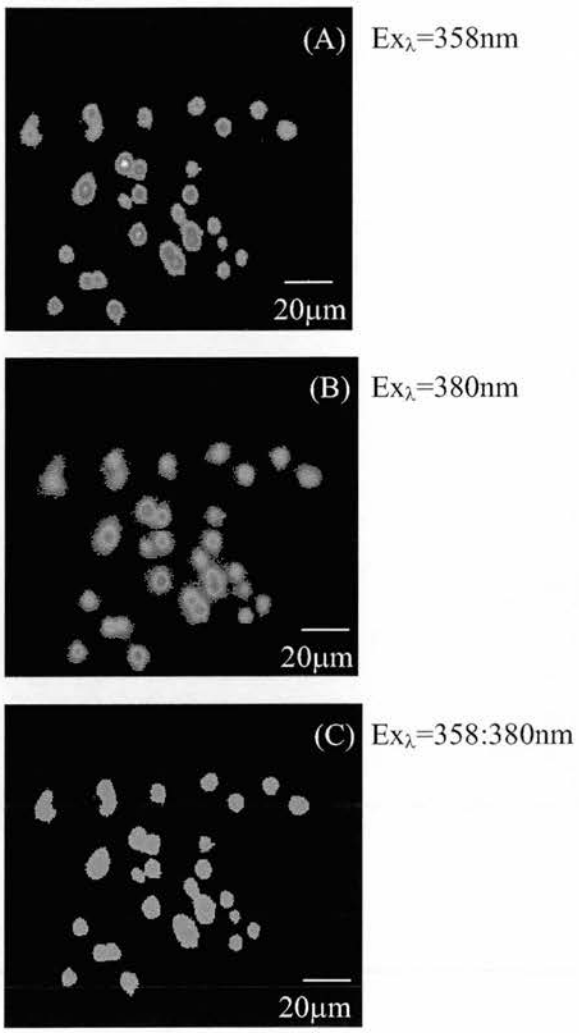


Figure 6.1. Fluorescent images of isolated human articular chondrocytes.

Chondrocytes were isolated from cartilage explants into 380mOsm.kg H2O-1 DMEM and incubated with fura-2 AM (5μM; 37°C; 30mins). The cells (contained within steel rings) were placed onto the stage a Nikon microscope and visualised through an x40 oil lens focused through the mid-plane. Images were acquired using a PTI Imagemaster™ system with the fura-2 excited alternately at 358nm (A; isobestic point, volume sensitive, $[Ca^{2+}]_i$ insensitive) and 380nm (B; volume sensitive, $[Ca^{2+}]_i$ sensitive) at 0.4Hz. A ratio image is also shown (C). The cells appeared rounded (as the above images are through a single optical plane the cells appear circular) with some heterogeneity in apparent cell size as seen by differences in cell diameter.

6.2.2 Regulatory Volume Decrease (RVD) in a Single Isolated Human Articular Chondrocyte.

In a typical experiment (Fig. 6.2), the resting fluorescence of fura-2 loaded chondrocyte was recorded under constant perfusion with the iso-osmotic 'control saline' (37°C; 380mOsm.kg H_2O^{-1}) for 3mins (V_{rest}). The perfusion solution was then switched to deliver a hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}; a complete change in osmolality in less than 90 seconds; see section 2.3.7) that resulted in a rapid fall in intracellular fluorescence. The maximum increase in volume (V_{max}) is shown by the minimum 358nm fluorescence value and in this example, this occurred at ~1.5mins following the hypo-osmotic challenge. Data were then recorded for the subsequent 10mins.

From the single cell example, it was possible to see that within the 10 minute recovery period there was complete RVD where the final volume (V_{final}) was not significantly different (Student's t-test; $p>0.05$) from that of the volume recorded in the 'control saline' (380mOsm.kg H_2O^{-1}). There was also a transient rise in $[Ca^{2+}]_i$ (shown by the rise in the 358:380nm ratio) that subsequently returned to basal levels during the experimental period. The maximum change in $[Ca^{2+}]_i$ occurred before the on-set of RVD and there it is possible that the rise is a mediator the volume-regulatory response.

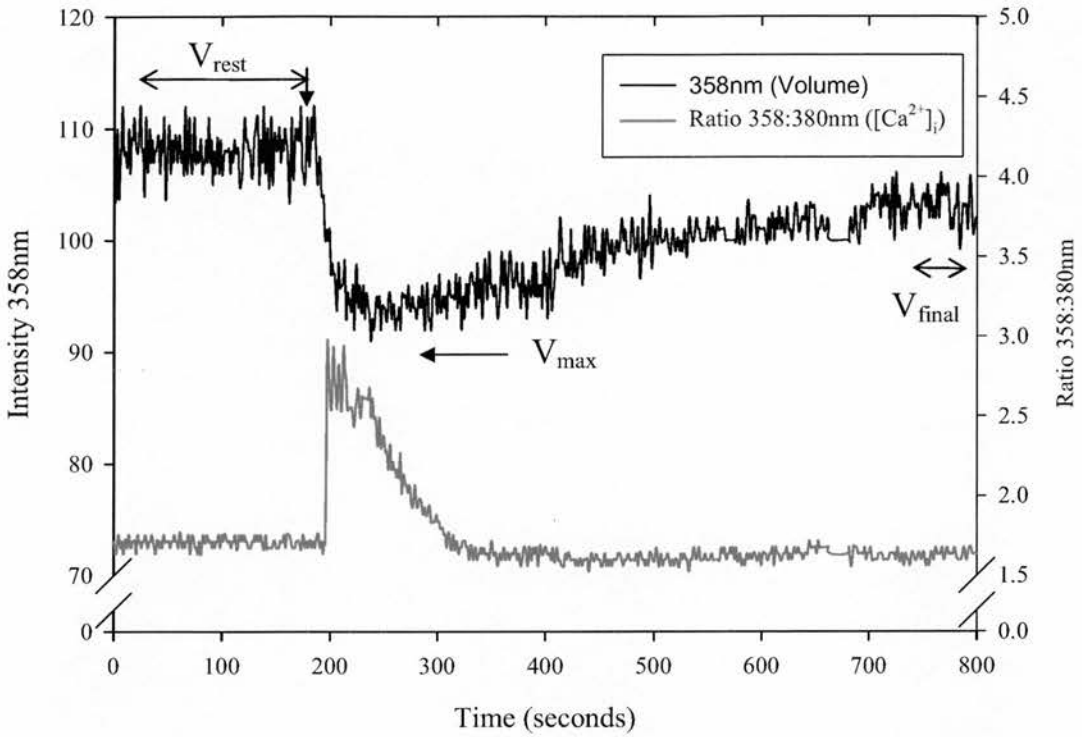


Figure 6.2. Example of a single isolated human chondrocyte volume regulating by RVD.

Freshly-isolated chondrocytes were incubated with fura-2AM (5μM; 30mins 37°C) in 380mOsm.kg H_2O^{-1} DMEM and visualised by fluorescent microscopy (see material and Methods) Resting intensity (V_{rest}) was recorded for 3mins under the constant perfusion of an iso-osmotic saline (380mOsm.kg H_2O^{-1}) and then a hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) was applied by perfusion (shown by the back vertical arrow). This resulted in a rapid fall 358nm fluorescence where the maximum increase in volume (V_{max}) is indicated by the minimum 358nm fluorescence; ~1.5min after the osmotic challenge. RVD then proceeded as shown by the recovery of fluorescence, where there was no significant difference ($p>0.05$) in the final volume (V_{final}) after 10mins. In response to the decrease in extracellular osmolality there was a transient rise in intracellular calcium (shown by the increase in the 358:380nm ratio) that then return the basal levels during the remainder of the experimental period. In this example, the rise in intracellular calcium did not appear to correlate with the onset of RVD. Data are expressed as a raw fluorescent trace, n= the fluorescent trace from 1 cell.

6.2.3 Examples of Volume and $[Ca^{2+}]_i$ Changes in Chondrocytes During a Hypo-Osmotic Challenge.

Chondrocytes from 'non-degenerate' areas of the tibial plateaux (areas that appeared macroscopically normal; see section 2.6.1) were isolated into 380mOsm.kg H_2O^{-1} DMEM, incubated with Fura-2 AM (5μM, 37°C; 30mins) and visualised by fluorescent microscopy as previously described (see Materials and Methods). Resting fluorescence was recorded under constant perfusion for 2mins and then a hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}; 43 %) was applied by perfusion. Data were subsequently recorded for 10mins.

In response to the osmotic challenge, some chondrocytes underwent RVD (Fig 6.4a & Fig 6.4b B) where at 10mins post-osmotic challenge (V_{final}), there was no significant difference (Student's t-test; $p>0.05$) in the 358nm fluorescence intensity when compared to the 'resting fluorescence' (V_{rest}). The decrease in extracellular osmolality initiated a transient rise in $[Ca^{2+}]_i$ in some cells that did not appear to correlate with the capacity for RVD. For example, in Fig 6.3a there was significant RVD and a rise in $[Ca^{2+}]_i$ where conversely, in Fig 6.3b there was also significant RVD but no change in $[Ca^{2+}]_i$. Interestingly, in the chondrocytes that did not volume regulate by RVD, there was still a transient rise in $[Ca^{2+}]_i$ in some cells (Fig 6.3c). Finally, in some cells there was neither RVD or a rise in $[Ca^{2+}]_i$ (Fig 6.3d). These data therefore highlight the heterogeneity of the chondrocyte response and the problems of working with a mixed population of cells.

In response to an osmotic challenge, some chondrocytes are able to volume regulate and it appeared that the capacity for RVD did not correlate with an increase in $[Ca^{2+}]_i$. This heterogeneity is mostly likely due to the fact that all experiments are performed using full-depth cartilage explants encompassing chondrocytes from all the cartilage zones. As mentioned in the previous chapters, it is possible that chondrocytes isolated from different zones respond to changes in osmolality in different ways. Although in the literature there are no specific studies on this. Previous work on pH-regulation on *in situ* bovine articular chondrocyte has shown that chondrocytes from different cartilage zones can have different responses to the same stimulus (O'Neill *et al.*, 2002), where chondrocytes from the SZ appear to pH regulate using a different set of membrane transporters when compared to MZ and DZ chondrocytes. This would further imply that a uniform response might be obtainable if these experiments were repeated using chondrocytes from isolated zones and not full depth explants.

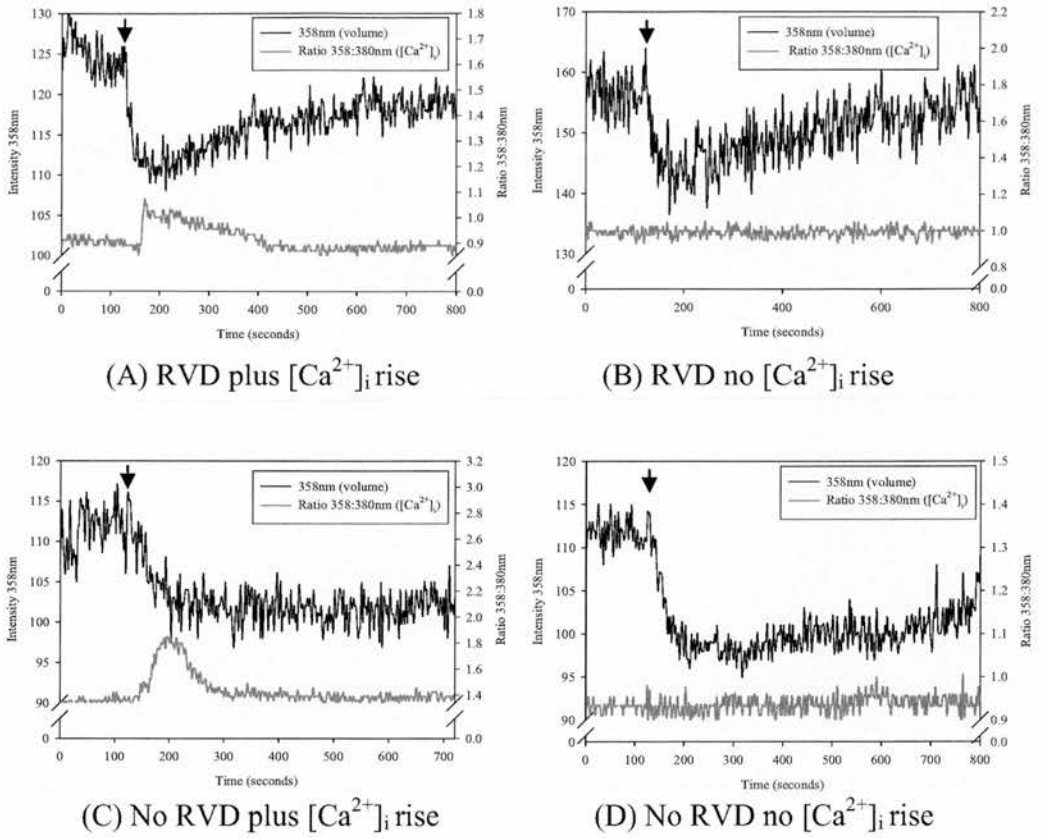


Figure 6.3. Heterogeneity of the RVD response in isolated human articular chondrocytes.

Chondrocytes from the tibial plateaux were isolated into 380mOsm.kg H_2O^{-1} DMEM, incubated with Fura-2 AM (5μM, 37°C; 30mins) and visualised by fluorescent microscopy as previously described (see Materials and Methods). Chondrocytes were perfused with an iso-osmotic saline for 2mins and then a 43 % hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) was applied by perfusion (shown by the black arrow). In response to the decrease in extracellular osmolality, some chondrocytes were able to regulate their volume by RVD and in some instances there was a rise in $[Ca^{2+}]_i$ (Fig A & B). Conversely, other chondrocytes did not perform RVD there was still a rise in $[Ca^{2+}]_i$ (Fig (C & D). These data show the heterogeneous response of the chondrocyte population where there does not appear to be a correlation between changes in $[Ca^{2+}]_i$ and the capacity for RVD. Data are expressed as raw fluorescence traces, N=4 knees (grade zero), n=4 cells.

6.2.4 RVD in Chondrocytes Isolated From 'Degenerate' and 'Non-Degenerate' Cartilage.

6.2.4.1 Pooled RVD for Isolated Human Articular Chondrocytes.

The hypothesis that chondrocytes isolated from 'degenerate' cartilage have an impaired RVD response was tested by exposing chondrocytes isolated from regions of 'non-degenerate' and 'degenerate cartilage' cartilage to a decrease in extracellular osmolality (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) and comparing the changes in volume and any regulation that occurred. Experiments were performed on cartilage explants excised from the tibial plateaus (having been graded according to the level of degeneration; see Materials and Methods) and the chondrocytes isolated into 380mOsm.kg H_2O^{-1} DMEM. Isolated chondrocytes were incubated with Fura-2 AM (5μM, 37°C; 30mins) and visualised by fluorescent microscopy as previously described (see Materials and Methods).

In response to the decrease in extracellular osmolality there was no significant difference ($p>0.05$) in the extent of maximal swelling (V_{max}) between chondrocytes isolated from 'non-degenerate' or 'degenerate' cartilage with maximal volume changes of 10.85 ± 0.17 % and 11.12 ± 0.1 % respectively (n=18 joints N=471 cells; samples of non-degenerate and degenerate cartilage were removed from each). Data were then plotted using semi-log plots (see Materials and Methods) and the rate of RVD calculated (expressed as a $t½$) by linear regression ($r^2>0.9$) and extrapolation (see

Materials and Methods). When comparing the rate of RVD, there was no significant difference (Student's t-test; $p>0.05$) between the $t_{1/2}$ values for 'non-degenerate' ($t_{1/2}$ = of 6.3 ± 1.9mins) and 'degenerate' ($t_{1/2}$ = of 6.1 ± 0.9 mins) experimental groups. Further supporting the fact there were no difference in the RVD response, the extent of RVD was calculated (expressed as a percentage reduction from maximal swelling) and compared between each experimental group. In chondrocytes isolated from 'non-degenerate' cartilage, and after 10mins post-osmotic challenge, volume had recovered by 69.5 ±6.4 %. In chondrocytes isolated from 'degenerate' cartilage, and after 10mins post-osmotic challenge, there had been 61.6 ± 6.6 % RVD; there was no significant difference ($p>0.05$; Table 6.1).

Despite the fact no differences were found in the maximal swelling, rate or extent of volume regulation in chondrocytes showing RVD, these data do not show if the percentage of chondrocytes from 'degenerate' cartilage able to perform RVD differed compared to 'non-degenerate' controls. When comparing the percentage of chondrocytes that performed RVD (see Materials and Methods), no significant difference ($p>0.05$) was found, with 26.0 ± 5.1 % of chondrocytes from 'non-degenerate' cartilage showing RVD and 24.8 ± 5.7 % of chondrocytes from 'degenerate' cartilage (n=18 joints, N=471 cells; Table 6.1).

The percentage of chondrocytes responding to a hypo-osmotic challenge with RVD is low when compared to responding chondrocytes isolated from bovine articular cartilage (~60-70 % of cells responding). When examining the human chondrocyte data for

responding cells per joint, there were some experiments where no chondrocytes underwent RVD in both 'non-degenerate' and 'degenerate' experimental groups. This heterogeneity in response is shown in the box plot (Fig. 6.4) where the range for each data set indicates a large spread. To give a truer representation of the parent population, experiments where there was no RVD were kept during sample analysis and therefore the apparent lower number of chondrocytes able to perform RVD. The fact that in these experiments the chondrocytes hydrolysed the fura-2 AM bond to yield the fluorescent form of fura-2, and remained attached to the coverslips during the experimental period would imply that the cells are viable and that the lower RVD response was not due to a high proportion of dead cells. When comparing these two experimental groups using an f-test (to examine differences in variance) and a t-test (probability test) no significant differences were found ($p>0.05$; n=18 joints; N=471 cells).

It was then tested to see if there were any differences in maximal swelling between 'responding' and 'non-responding' for both experimental groups. There was no significant difference (Student's t-test; $p>0.05$) when comparing cell swelling between 'responding' and 'non-responding' chondrocytes for both 'non-degenerate' and 'degenerate' experimental groups. In 'non-degenerate' cartilage, the chondrocytes that underwent RVD had a maximal volume increase of 9.88 ± 0.17 % compared to 10.85 ± 0.17 % in cells that did undergo RVD. In chondrocytes from 'degenerate' cartilage there was a maximal volume change of 9.42 ± 0.6 % in cells that did not undergo RVD compared to 11.12 ± 0.1 % in cells that did perform RVD (N=18 joints, n=471 cells; Table 6.1).

These data show that chondrocytes isolated from 'non-degenerate' and 'degenerate' cartilage have the capacity for RVD in response to a direct hypo-osmotic challenge. No significant differences were found when comparing the RVD response between each experimental group and therefore, based upon these data; the initial hypothesis that chondrocytes originating from 'degenerate' cartilage have an impaired RVD response was not supported by these experiments. It is possible that there are differences in the chondrocyte cell population as a result of isolation from the matrix and this will be discussed in section 6.4.0.

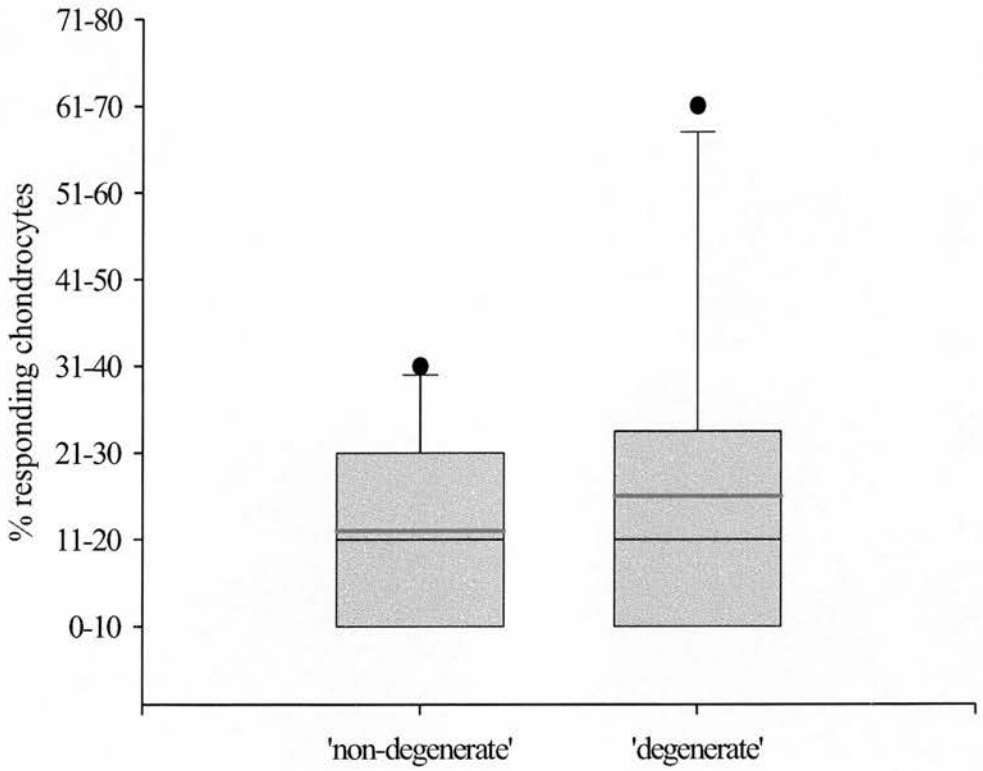


Figure 6.4. The heterogeneous RVD response in isolated human articular chondrocyte.

Chondrocytes from the tibial plateau were isolated into 380mOsm.kg H_2O^{-1} DMEM, incubated with fura-2 AM (5μM, 37°C; 30mins) and visualised by fluorescent microscopy as previously described (see Materials and Methods). Chondrocytes were perfused with an iso-osmotic saline for 2mins and then a 43 % hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) was applied by perfusion. The percentage of chondrocytes volume regulating by RVD was calculated for each experiment (one experiment = one tibial plateau) according to the experimental grouping 'non-degenerate' and 'degenerate' (see Materials and Methods). The number of chondrocytes able to volume regulate was heterogeneous for each experiment within and outwith both experimental groups. The box plots show large whiskers and outliers indicating a large spread of data. The red bar indicates the sample mean. When comparing the distribution and range (using F & T tests) there was no significant difference ($p>0.05$). Data are expressed as mean ± s.e.m, N=18 joints (areas of degenerate & non-degenerate removed from each), n=471 cells. Black dots indicate data 'outliers' from each experimental group

	Chondrocytes isolated from		
	'Non-degenerate'	**'Degenerate'**	**Significant?**
Maximal volume change (%)	10.85 ± 0.17	11.2 ± 0.10	N/S
% Cells showing RVD	26.4 ± 6.20	35 ± 6.4	N/S
Rate of RVD (t ½; mins)	6.3 ± 1.9	6.1 ± 0.9	N/S
Extent of RVD after 10mins (%)	69.5 ± 6.4	61.6 ± 6.6	N/S

Table 6.1. RVD in chondrocytes from 'non-degenerate' and degenerate cartilage.

Chondrocytes from the tibial plateau were isolated into 380mOsm.kg H_2O^{-1} DMEM, incubated with Fura-2 AM (5μM, 37°C; 30mins), visualised by fluorescent microscopy and RVD experiments performed as previously described (see Materials and Methods). RVD was compared between chondrocytes isolated from areas of 'non-degenerate' and 'degenerate' cartilage to test the hypothesis that chondrocytes from 'degenerate' cartilage have an impaired RVD response. When comparing the extent of cell swelling the rate of RVD and the percentage of responding no significant difference were found (Student's t-test; p>0.05). These data show the hypothesis that chondrocytes originating 'degenerate' cartilage have an impaired RVD response is invalid based upon these data. Data are expressed as mean ± s.e.m, N=18 joints (areas of degenerate & non-degenerate removed from each), n=471 cells.

6.2.4.2 Changes in $[Ca^{2+}]_i$ in Human Isolated Articular Chondrocytes.

In response to the decrease in extracellular osmolality (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) there was a rise in intracellular calcium ($[Ca^{2+}]_i$) in some cells that subsequently returned to basal levels during the 10 minute experimental period. To examine potential differences in the $[Ca^{2+}]_i$ response between the degenerate and non-degenerate experimental groups, the changes in $[Ca^{2+}]_i$ were standardised to values recorded during the initial 'resting period' (V_{rest}; see Materials and Methods) and expressed as a percentage change relative to these basal levels. These values were then correlated to 'responding' (cells showing greater than 50% RVD) and 'non-responding' (cells showing less than 50% RVD) chondrocytes and statistical analysis performed using a Student's t-test and Chi-squared (χ^2), analysis.

When comparing the $[Ca^{2+}]_i$ in chondrocytes isolated from 'non-degenerate' and 'degenerate' cartilage, there were no significant differences ($p>0.05$) in the maximal rise with changes of 19.94 ± 3.45 % and 22.21 ± 5.42 % recorded respectively (n=18 joints, N=471 cells; Table 6.2). In chondrocytes isolated from 'non-degenerate' cartilage, 48.21 ± 8.72 % of cells demonstrated a greater than 10 % rise in $[Ca^{2+}]_i$ and 27.99 ± 7.77 % exhibited a rise greater than 20 % over basal levels. In chondrocytes originating from 'degenerate cartilage', 54.13 ± 7.02 % had a rise in $[Ca^{2+}]_i$ greater than 10 % rise and 41.03 ± 7.19 % had a rise greater than 20 % over basal levels. When using a Student's t-test to compare these $[Ca^{2+}]_i$ changes, no significant difference ($p>0.05$) between the experimental groups were found (n=18 joints, N=471 cells; Table 6.2).

Having established no significant differences in the rise in $[Ca^{2+}]_i$ between chondrocytes isolated from 'non-degenerate' and 'degenerate', it was subsequently tested to see if the rise in $[Ca^{2+}]_i$ corresponded with the capacity for RVD. As previously described, the changes in $[Ca^{2+}]_i$ were correlated to 'responding and 'non-responding' experimental groups (see Materials and Methods). There was no significant difference ($p>0.05$) between the maximal $[Ca^{2+}]_i$ rises for both 'responding' and 'non-responding' chondrocytes for both 'non-degenerate' and 'degenerate' experimental groups (Table 6.2). In chondrocytes isolated from 'non-degenerate' cartilage, that did not volume regulate, there was a maximal $[Ca^{2+}]_i$ rise of 15.27 ± 3.17 % compared to 19.94 ± 3.45 % in cells that did undergo RVD. In chondrocytes isolated from 'degenerate' cartilage there was a maximal $[Ca^{2+}]_i$ change of 19.82 ± 2.37 % in cells that did not undergo RVD compared to 22.21 ± 5.42 % in cells that did perform RVD (n=18 joints, N=471 cells).

When comparing the percentage of chondrocytes that underwent RVD with a rise in $[Ca^{2+}]_i$ to those that under went RVD without rise in $[Ca^{2+}]_i$ outwith an experimental groups there was no significant difference ($p>0.05$; Table 6.5). Conversely, for both experimental groups ('non-degenerate' and 'degenerate'), the percentage of cells that did not show RVD but a rise in $[Ca^{2+}]_i$ was significantly greater ($p<0.05$) to those that did show RVD. Overall, using a Chi-squared test (χ^2), there was no significant difference between the calcium changes for 'non-degenerate' and degenerate chondrocytes ($p>0.05$).

These data show that in response to a decrease in extracellular osmolality there was a transient rise in $[Ca^{2+}]_i$ that returned to basal levels during the experimental period. There was no significant difference in the $[Ca^{2+}]_i$ response between chondrocytes isolated from 'non-degenerate' and 'degenerate' cartilage with respect to the magnitude or the number of responding cells. There was no correlation between the rise in $[Ca^{2+}]_i$ and the capacity for RVD although there was a significant increase ($p<0.05$) in number of chondrocytes that did not show RVD but a rise in $[Ca^{2+}]_i$ compared to that showed both RVD and a rise in $[Ca^{2+}]_i$ for both experimental groups.

	'Non-degenerate'		'Degenerate'	
Experimental group	**RVD**	**No RVD**	**RVD**	**No RVD**
Maximal change in volume (%)	10.85 ± 0.17	9.88 ± 0.17	11.2 ± 0.10	9.42 ± 0.6
Maximal $[Ca^{2+}]_i$ rise	19.94 ± 3.45	15.27 ± 3.17	22.2 ± 5.42	19.82 ± 2.37
% Cells $[Ca^{2+}]_i$ rise (>10 %)	13.47 ± 3.29	34.73 ± 8.26 *	14.09 ±4.40	40.72 ± 6.01 *
% Cells $[Ca^{2+}]_i$ rise (<10 %)	12.53 ± 3.21	39.26 ± 7.65 *	10.67 ±4.00	35.24 ± 4.61 *

Table 6.2. RVD and changes in $[Ca^{2+}]_i$ in chondrocytes from 'non-degenerate' and 'degenerate' articular cartilage.

*RVD was measured in chondrocytes isolated from areas of 'non-degenerate' and 'degenerate' cartilage in response to a 43 % osmotic challenge. Subsequently, the RVD data were divided into 'responding' and 'non-responding' experimental groups (see Materials and Methods and the changes in $[Ca^{2+}]_i$ calculated as a percentage change from 'resting' levels. No significant difference ($p>0.05$) was found in cell swelling or the maximal $[Ca^{2+}]_i$ rise within or outwith the experimental groups. There was also no significant difference ($p<0.05$) in the percentage cells that underwent RVD and had a rise in $[Ca^{2+}]_i$ between 'non-degenerate' and 'degenerate' experimental groups. Interestingly, a significantly greater percentage ($p<0.05$; Students t-test; shown as *) of 'non-responding' chondrocytes had a rise in $[Ca^{2+}]_i$ when compared to 'responding' chondrocytes for both experimental groups. Data are expressed as mean ± s.e.m, N=18 joints, n=471 cells.*

Treatment	Maximal $[Ca^{2+}]_i$ rise	% Cells $[Ca^{2+}]_i$ rise (>10 %)	% Cells $[Ca^{2+}]_i$ rise (>20 %)
'Non-degenerate'	19.94 ± 3.45	48.21 ± 8.72	27.99 ± 7.77
Degenerate	22.21 ± 5.42	54.13 ±7.02	41.03 ± 7.19
50μM REV 5901	7.48 ± 1.12	14.95 ± 10.89***	0.00 ± 0.00 ***
100μM Gadolinium	12.83 ± 0.62	15.85 ± 4.46***	8.70 ± 3.43***
10μM Tamoxifen	12.37 ± 0.20	11.11 ± 11.11***	0.00 ± 0.00***

Table 6.3. Percentage of chondrocytes responding to a 43 % osmotic challenge with a rise in $[Ca^{2+}]_i$.

RVD and changes in $[Ca^{2+}]_i$ (calcium changes calculated as a percentage ratio change) were measured in chondrocytes isolated from areas of 'non-degenerate' and 'degenerate' cartilage in response to a 43 % osmotic challenge. The effects of REV 5901 (inhibitor of the bovine 'osmolyte channel' and LTD_4 antagonist), gadolinium (inhibitor of stretch channels and tamoxifen (PLC inhibitor and non-selective Cl^- channel blocker) were also studied. It was found that the overall number of chondrocytes showing a rise in calcium (and the maximal change) was attenuated by the addition of REV 5901 or gadolinium. REV 5901 completely inhibited the 20% intracellular calcium rise $p<0.001$). There was no significant difference ($p>0.05$) in the maximal $[Ca^{2+}]_i$ rise or the overall percentage of chondrocytes responding when comparing degenerate and non-degenerate experimental groups. The data shown for the REV 5901 and the gadolinium experimental groups are collective for degenerate and non-degenerate cartilage as no significant difference was found between the two (data not shown). Preliminary tamoxifen data would suggest it is a good inhibitor of the rise in $[Ca^{2+}]_i$. Data are expressed as mean ± s.e.m, N=18 joints, n=471 cells.

6.2.5 REV 5901 does not block human chondrocyte RVD.

It has previously been shown that the addition of REV 5901 (a specific antagonist of the LTD_4 Leukotrine receptor (Aharony *et al.*, 1986); non-specific inhibitor of the 'osmolyte' channel) inhibited RVD in bovine articular chondrocytes (~95 %; Hall & Kerr, 2000; Kerrigan & Hall, 2000). Here, the affect of REV 5901 on human articular chondrocyte RVD was subsequently tested. Experiments were performed as previously described (see Materials and Methods) using fura-2 loaded chondrocytes isolated from the tibia plateau into 380mOsm.kg H_2O^{-1} DMEM. Chondrocytes were incubated with REV 5901 (50μM; 30mins; 37°C) and standard RVD experiments were performed. REV 5901 was also present in all experimental salines. Cells were perfused with an iso-osmotic saline (380mOsm.kg H_2O^{-1}) for 2mins and then a hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) was applied by perfusion.

In response to the decrease in extracellular osmolality there was an increase in cell volume as indicated by a fall in cellular fluorescence (recorded at 358nm; emission of 510nm) as previously described (see section 6.2.2). There was no significant difference ($p>0.05$; Student's t-test) in the maximal cell swelling with changes in fluorescence of 11.32 ± 1.04 % (control chondrocytes) and 10.85 ± 1.70 % (REV 5901 treated chondrocytes). Chondrocytes then proceeded to undergo RVD where there was no significant difference ($p>0.05$) in either the rate ($t½$ = 9.1 ± 1.3mins) or the percentage volume recovered (82.71 ± 12.41 %) between experimental and control groups (Table 6.3; Fig 6.5).

Despite REV 5901 not having an effect on RVD, it was still possible that the changes in $[Ca^{2+}]_i$ associated with a decrease in extracellular osmolality may be sensitive to the drug. Data were recorded and the change in $[Ca^{2+}]_i$ expressed as a percentage of cells showing a standardised rise greater than 10 % or 20 % over basal levels (see materials and methods). A 30min pre-incubation with REV 5901 resulted in an significant attenuation ($p<0.05$) in the maximal rise in $[Ca^{2+}]_i$ and significantly decreased ($p<0.001$) the percentage of chondrocytes showing a rise in $[Ca^{2+}]_i$ at the 10 % level. The drug completely inhibited the number of chondrocytes responding with a rise greater than 20 % over basal levels (Table 6.3; n = 18 joints, N = 612 cells).

These data show that RVD in isolate human articular chondrocytes was not inhibited by REV 5901. When compared to controls, RVD proceed 'normally' with no significant deviation in maximal cell swelling, rate or percentage recovery. Conversely, the rise in $[Ca^{2+}]_i$ was attenuated and the number of chondrocytes showing a $[Ca^{2+}]_i$ rise was significantly decreased ($p<0.001$) at the 10% level and the response abolished at the 20% level ($p<0.001$).

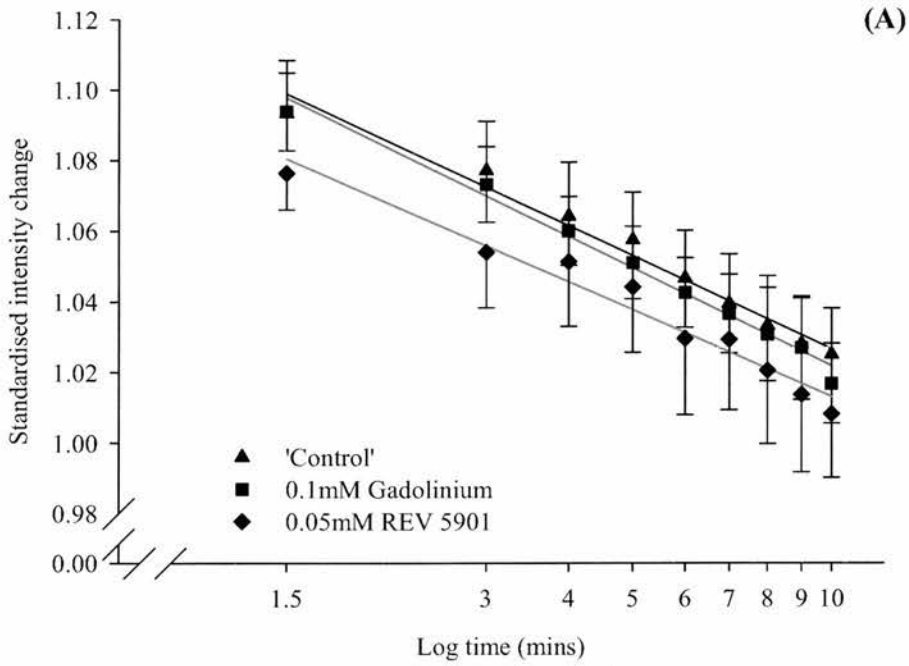


Figure 6.5. Volume regulation and changes in $[Ca^{2+}]_i$ in human articular chondrocytes in the presence of gadolinium or REV 5901.

RVD was measured in isolated chondrocytes in response to a 43% hypo-osmotic challenge and changes in volume and $[Ca^{2+}]_i$ were calculated as a standardised change relative to 'resting' levels (see materials and methods). (A) Volume data were plotted using a semi-log scale and regression analysis performed (see materials and methods). There was no significant difference ($p>0.05$; Students t-test) in either the maximal swelling, the rate of RVD or the final volume recovered between any of the experimental groups. Data are expressed as mean ± s.e.m, N = 18 joints, n = 719 cells.

6.2.6 Gadolinium did not block human chondrocyte RVD.

In 2D cultured human articular chondrocytes it has been shown that intermittent cyclical pressure (0.33Hz; 16Pka; 20mins) hyperpolarises the cell mediated by a stretch-induced, gadolinium-sensitive low-conductance, apamin-sensitive, Ca^{2+}-activated K^{+} channel (Wright *et al.*, 1996; Wright & Salter, 1996). The calcium rise is mediated via an influx (from the extracellular environment) and inhibited by a pre-incubation with 10μM gadolinium chloride (Gd^{3+}). As it has been shown, that Gd^{3+} inhibits mechanotransduction in response to fluid flow and membrane deformation (Yellowley *et al.*, 1997; Guilak *et al.*, 1999b; Yellowley *et al.*, 1999) and that RVD could be a response to membrane stretch, here, the effect of Gd^{3+} on chondrocytes RVD was tested.

Chondrocytes were pre-incubated with gadolinium chloride (dissolved in saline; 100μM; 30mins; 37°C) and the standard RVD experiments were performed. Gadolinium was also present in all experimental salines at the same working concentration. Cells were perfused with an iso-osmotic saline (380mOsm.kg H_2O^{-1}) for 2mins and then a hypo-osmotic challenge (380mOsm.kg H_2O^{-1} to 220mOsm.kg H_2O^{-1}) was applied.

In response to the decrease in osmolality there was an increase in cell volume where there was no significant difference ($p>0.05$; Student's t-test) in the standardised cell swelling between control and experimental groups with changes of 11.32 ± 1.04 % and 9.38 ± 1.10 % respectively. RVD then proceeded linearly with no significant difference

($p>0.05$; when compared to controls) in either the rate of RVD ($t½ = 5.9 \pm 0.79$), the percentage of cells responding (46.46 ± 14.07 %) or the percentage volume recovered during the 10 minute experimental period (81.74 ± 7.39 %; Table 6.2; N = 18 joints, n = 653 cells).

As previously observed in bovine articular chondrocytes (section 3.2.6), the decrease in extracellular osmolality initiated a transient rise in $[Ca^{2+}]_i$ in some cells. Even though in bovine articular chondrocytes the rise was insensitive to Gd^{3+}, it was possible that the rise in $[Ca^{2+}]_i$ in human articular chondrocytes was mediated though a gadolinium-sensitive channel. Therefore the effect of 100μM Gd^{3+} on the rise in $[Ca^{2+}]_i$ in response to the osmotic challenge was studied. Data were recorded and the change in $[Ca^{2+}]_i$ expressed as a percentage of cells showing a standardised rise greater than 10 % or 20 % over basal (see material and methods). There was an attenuation in the maximal $[Ca^{2+}]_i$ rise with a standardised rise of 10.07 ± 3.89 % compared to 19.94 ± 3.45 % in chondrocytes from 'non-degenerate' cartilage and 22.21 ± 5.42 % from chondrocytes isolated from 'degenerate' cartilage. There was a significant decrease ($p<0.01$) in the percentage of chondrocytes responding at both the 10 % and the 20 % levels with 15.85 ± 4.46 % and 8.70 ± 3.43 % of chondrocytes responding respectively (Table 6.3; n = 18 joints, N = 653 cells).

The inhibitory effect of Gd^{3+} may have related to the grade of cartilage from which the chondrocytes were isolated. Therefore, the effects of Gd^{3+} were related to the two experimental groups. In chondrocytes isolated from 'non-degenerate' (grades 0 and 1) cartilage 47.69 ± 14.35% of cells underwent RVD compared to 33.86 ± 17.32% of cells

isolated from 'degenerate cartilage' (grades 2 and 3); no significant difference (Student's t-test; $p>0.05$). Gd^{3+} inhibited the rise in $[Ca^{2+}]_i$ in both 'non-degenerate' and 'degenerate' experimental groups where in controls 51.33 ± 8.55% and 52.29 ± 6.42 of cells responded. In response to the pre-incubation with Gd^{3+}, the percentage of cells responding was significantly reduced ($p<0.05$) to 18.21 ± 5.83% and 14.29 ± 7.43 respectively (Fig. 6.6).

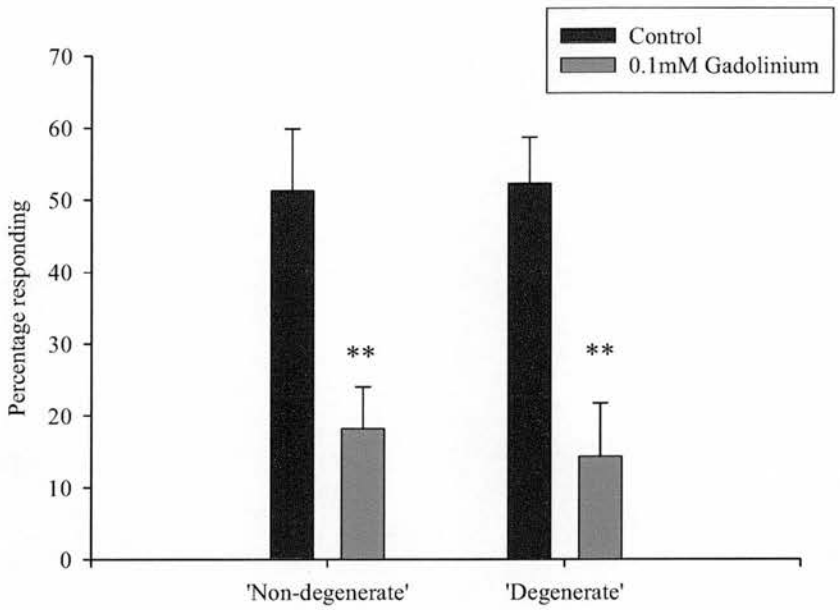


Figure 6.6. The effect of 100μM Gadolinium on the hypo-osmotic induced rise in $[Ca^{2+}]_i$ in human articular chondrocytes.

RVD was studied in human articular chondrocytes in response to a 43% hypo-osmotic challenge in a 1mM calcium saline. The effect of Gd^{3+} on the RVD response (see section 6.2.6) and the rise in $[Ca^{2+}]_i$ was tested. Prior to an experiment, cells were incubated with Gd^{3+} (30mins; 37°C) and the experiments performed as previously described. The decrease in extracellular osmolality resulted in a rise in $[Ca^{2+}]_i$ that was not significantly different between chondrocytes isolated from 'non-degenerate' or 'degenerate' cartilage. The rise in $[Ca^{2+}]_i$ was inhibited by 100μM Gd^{3+} with equal efficacy in both experimental groups. Data are expressed as mean ± s.e.m. n= 18 joints N= 653 cells.

These data show that the RVD response in isolated human articular chondrocytes was not sensitive to a pre-incubation with 100μM gadolinium and there implies that RVD may not be mediated via stretch-sensitive ion channel. Conversely, the transient rise in $[Ca^{2+}]_i$ was sensitive to gadolinium and therefore could be mediated through a stretch-sensitive channel. The fact that the rise in $[Ca^{2+}]_i$ was significantly inhibited but RVD was not would imply that RVD in human articular chondrocytes is not dependent upon the $[Ca^{2+}]_i$ rise. The was no correlation between the inhibitory effect of Gd^{3+} and the degeneration of cartilage and therefore the rise in $[Ca^{2+}]_i$ may be mediated by the same pathway from chondrocytes in both experimental groups.

6.3.0 Results Summary.

The work presented in this chapter has shown that freshly-isolated human articular chondrocytes isolated from 'degenerate' and 'non-degenerate' cartilage are capable of RVD following a hypo-osmotic deformation due to a decrease in extracellular osmolality. There was no difference in the RVD response between the two experimental groups nor was there a difference in the changes in the $[Ca^{2+}]_i$ rise observed in response to the osmotic challenge. Unlike bovine articular chondrocytes (BAC's), RVD was not inhibited by a pre-incubation with 50μM REV 5901 although the maximum calcium $[Ca^{2+}]_i$ was attenuated and the percentage of chondrocytes exhibiting the rise significantly decreased. Conversely, as in BAC's, RVD was not inhibited by the pre-incubation with 100μM gadolinium chloride although unlike BAC's the maximal $[Ca^{2+}]_i$ rise was attenuated and the percentage cells responding with a $[Ca^{2+}]_i$ rise was significantly decreased.

6.4.0 Chapter Discussion

The data presented in this chapter have shown that there were no differences in the capacity for RVD between chondrocytes isolated from 'non-degenerate' or degenerate cartilage (Table 6.1; Fig. 6.4). Furthermore, RVD was not inhibited by the bovine chondrocyte 'osmolyte channel' inhibitor REV 5901 (Fig. 6.4) or by a pre-incubation with Gd^{3+} (Fig. 6.5). The decrease in extracellular osmolality resulted in a transient rise in $[Ca^{2+}]_i$ that did not appear to correlate with the capacity for RVD or the cartilage grade of degeneration (Table 6.2).

In response to the 43% hyper-osmotic challenge, there were no differences in the maximal change in cell volume, the rate or RVD or the percentage cells responding between chondrocytes isolated from 'degenerate' or 'non-degenerate' articular cartilage (Table 6.1). This would therefore suggest that the increase in chondrocyte volume observed in OA cartilage (Bush & Hall, 2003) was not the result of an impaired RVD response. This is a significant finding as chondrocytes within 'degenerate' cartilage have a larger cell volume when compared to chondrocytes in 'non-degenerate' cartilage. Furthermore, the increase in volume was greater than that predicted by passive swelling due to an increase in cartilage hydration (Bush & Hall, 2003) and therefore may be the result of a altered cellular response. Therefore, based on these data alone, another reason needs to be found to explain the increase in cell volume. This last point will be discussed further on.

Another significant finding of this study was that Gd^{3+} did not inhibit the RVD (Fig. 6.5) which contrasts with the fact that is has been shown to block pressure induced strain in human articular chondrocytes (Wright and Salter, 1996). Pressure induced strain activates a $\alpha 5\beta 1$ integrin receptor-ligand complex which can be inhibited by actin depolymerisation or 10μM Gd^{3+} (Wright & Salter, 1996). If the same pathway mediated RVD and the stretch response, one would anticipate that Gd^{3+} would inhibit both. The fact that the RVD presented here was insensitive to Gd^{3+} suggests that the same signal transduction pathway does not mediate both responses. Conversely, the hypo-osmotic transient rise in $[Ca^{2+}]_i$ was inhibit by a pre-incubation with Gd^{3+} therefore suggesting that the rise in calcium was mediated via a stretch-sensitive channel (Fig. 6.7; a predicted model for RVD in human articular chondrocytes). In articular chondrocytes, a stretch sensitive rise in $[Ca^{2+}]_i$ has also been observed in response to fluid flow and membrane deformation (Yellowley *et al.*, 1996; Yellowley *et al.*, 1997; Guilak *et al.*, 1999b) and therefore it is possible that the same pathway mediates the rise observed here. These data would therefore suggest that the rise in $[Ca^{2+}]_i$ is not a mediator of the RVD as human articular chondrocytes are able to volume regulate in the presence of Gd^{3+} despite the lack of a rise in calcium.

The inhibition of the $[Ca^{2+}]_i$ transient without any attenuation of the RVD response is an important observation, as previously it has not been possible to separate volume regulation and changes in $[Ca^{2+}]_i$. When correlating RVD and the percentage of cells showing a rise in $[Ca^{2+}]_i$, significantly more cells responded with a rise in $[Ca^{2+}]_i$ without showing significant RVD (Table 6.2). This would strongly suggest that the

calcium rise and RVD are two separate, contiguous events, both stimulated by cell swelling. It is not possible to rule out the possibility that the calcium rise is involved in RVD in some chondrocytes although as there was no decrease in the percentage of chondrocytes responding in the presence of Gd^{3+} this would therefore strongly suggest that the pathway is the same for all chondrocytes within human articular cartilage.

The fact that REV 5901 did not inhibit the RVD (Fig. 6.5) may imply that RVD by human articular chondrocytes is not mediated by the same pathway as previously observed in bovine articular chondrocytes (Hall, 1995; Bush & Hall, 2000; Hall & Kerr, 2000; Kerrigan & Hall, 2000; Hall & Bush, 2001). However, REV 5901 inhibition is non-specific (Hall & Kerr, 2000), and as the current mode of inhibition remains unknown, it would be premature to suggest two different pathways for RVD. REV 5901 has an IC_{60} 6μM for lipoxygenase inhibition (Vaninwegen *et al.*) and a IC_{50} of 8 ± 4μM for bovine taurine effect inhibition (Hall & Bush, 2001). It is quite possible that a higher concentration of REV 5901 will attenuate the RVD response in human articular chondrocytes and this remains to be tested. Interestingly, the pre-incubation with REV 5901 significantly decreased the percentage of cells showing a transient rise in $[Ca^{2+}]_i$ in response to the osmotic challenge further suggesting that the $[Ca^{2+}]_i$ rise is not a mediator of the RVD response.

The data presented so far has strongly suggested that RVD in human articular chondrocytes is independent of a rise in $[Ca^{2+}]_i$ and not mediated by the $\alpha 5\beta 1$ integrin receptor-ligand complex. Further supporting the hypothesis that volume and stretch are

mediated by two separate pathways is preliminary data by Wright *et al.*, (2002). It was shown that REV 5901 inhibited the pressure-induced strain response in chondrocytes isolated from both 'non-degenerate' and 'degenerate' articular cartilage (Dr M.O. Wright personal communication 2002). One could speculate that REV 5901 is acting as an inhibitor of stretch-sensitive channels in human articular chondrocytes, and the calcium rise is mediated by a stretch-sensitive calcium channel. Therefore an inhibition of the $[Ca^{2+}]_i$ transient by either Gd^{3+} or REV 5901 will block the pressure-induced strain response. Furthermore, it is quite possible that RVD is not mediated by a rise in $[Ca^{2+}]_i$, a change in membrane potential, or the activation of SK K^+ channels mediated by the $\alpha5\beta1$ integrin receptor-ligand complex.

Having reviewed the experimental data presented in this chapter, a few experimental considerations need to be addressed. It was possible that only viable cells would have survived the isolation process and therefore it is conceivable that cells lacking RVD did not survive. This might skew the overall inference on the capacity for *in* situ RVD. For example, assuming most chondrocytes in 'non-degenerate' cartilage have the capacity for RVD whereas only a few from 'degenerate' cartilage do, upon isolation it is possible that those cells that are not able to volume regulate will die. Chondrocytes are isolated into a medium of 380mOsm, whereas the osmolality of cartilage follows the FCD of the tissue (Maroudas, 1980) and therefore chondrocytes do not reside in an environment of equal osmolality. Chondrocytes from the SZ will effectively receive a hyper-osmotic challenge and those in the DZ a hypo-osmotic challenge upon release from the matrix. These differences may influence the overall isolated population. To resolve this

problem, and to be sure that the RVD response is the same between chondrocytes from 'non-degenerate' and 'degenerate' cartilage is similar *in situ* confocal analysis of RVD would need to be performed.

Another consideration relates to the thinning of cartilage during the progression of OA (Grushko *et al.*, 1989; Buckwalter & Mankin, 1997b). This would suggest that chondrocytes in the 'non-degenerate' experimental group would encompass cells from all three major cartilage zones whereas cells in the 'degenerate' group may primarily be from the MZ and DZ (Fig 2.6.3). Furthermore, it was also possible that there was increased cell death in chondrocytes from 'degenerate cartilage' in the cartilage that had not been lost due to erosion. In the isolated chondrocyte preparations, cell viability was often checked by trypan blue and usually ~95% of cells were viable. This, in conjunction with the fact that the chondrocytes were able to hydrolyse the fura-2 AM to fura 2, indicates that the cells studied were viable. In order to try to limit these potential problems during analysis, data from all joints were used during analysis even if no RVD was seen in any of the cells studied. To study these possibilities, *in vivo* confocal studies of human articular cartilage using calcein (an indicator of living cells) and propidium iodide (an indicator of dead cells) could be used.

The fact that chondrocytes isolated from 'degenerate' cartilage do not have an impaired RVD response would suggest a different mechanism for the increase in chondrocyte volume during the progression of OA. One possibility would include the activation of

the RVI response and the consequential influx of osmolytes resulting in cell swelling. Chondrocytes have been shown to express the NKCC cotransporter (see section 4.2.5) whose functionality has been shown by bumetanide-sensitive ^{86}Rb influx experiments (Hall *et al.*, 1996a, 1996b). The activation of the NKCC under iso-osmotic conditions would bring about cell swelling and it has been shown in porcine articular cartilage that the NKCC is functional and able to mediate the 'post RVD-RVI' response (Errington *et al.*, 1997). Interestingly no increase in expression between the 'non-degenerate' and the 'degenerate' state has been shown (Trujillo *et al.*, 1999) although this does not rule out the possibility of increased activity.

In summary, it does not appear that the RVD response is lost in chondrocytes isolated from 'degenerate' cartilage. The decrease in extracellular osmolality, initiated a transient rise in $[Ca^{2+}]_i$ that was inhibited by both REV 5901 and Gd^{3+} although neither drug inhibited RVD. This would therefore suggest that the rise in $[Ca^{2+}]_i$ may not be involved in the RVD response and may be implicated in another cellular process. Furthermore, the mechanism of RVD may be separate to that of membrane stretch implying two different pathways although with some overlap with respect to the rise in $[Ca^{2+}]_i$ mediated by a stretch-induced, gadolinium-sensitive calcium channel. A stretch insensitive rise in $[Ca^{2+}]_i$ has been observed in newborn rat cardiomyocytes (Taouil *et al.*, 1998) and in EAT cells it has been shown that a calcium-insensitive, nonselective cation channel activated by a decrease in osmolality could not be activated by membrane stretch (Christensen & Hoffmann, 1992). Furthermore, in clonal N1E115 neuroblastoma cells, only a variable block of RVD was achieved in the presence of

Gd^{3+} or amiloride suggesting that the response was not dependent upon stretch-sensitive channels (Lippmann *et al.*, 1995).

Based on the data presented in this chapter and in relation to the pressure-induced strain response described by Milllward-Sadler *et al.*, (1999) Figure 6.7 suggests a possible pathway for RVD in human articular chondrocytes. A change in osmolality initiates a rise in $[Ca^{2+}]_i$ mediated by a stretch-sensitive calcium channel that can be inhibited by REV 5901 or Gd^{3+}. This may then lead to the activation of the low-conductance apamin-sensitive K^+ channel that may be involved in the RVD response. The decrease in osmolality also activates a volume-sensitive, calcium-insensitive anion channel that is likely to be the primary mediator of the RVD response. Preliminary data suggests that RVD was inhibited by tamoxifen (although the drug is cytotoxic and more work is required dueo to the small n value) and may involve the PKC pathway. Furthermore, tamoxifen is a non-specific inhibitor and may be acting on another pathway. Conversely, membrane stretch also initiates a REV 5901/Gd^{3+} rise in $[Ca^{2+}]_i$ and the subsequent activation of the low-conductance apamin-sensitive K^+ channel via the $\alpha5\beta1$ integrin receptor-ligand complex.

It is still unclear how human articular chondrocytes regulate their volume in response to a decrease in extracellular osmolality and why there is an increase in cell volume in OA cartilage. Furthermore, the exact role for the hypo-osmotic induced $[Ca^{2+}]_i$ transient is still unclear although it does not appear to be involved in volume regulation. Further experiments are still required to elucidate mechanism for RVD, *in situ* confocal

experiments to ascertain cell viability as well as the relationship between the zone in which chondrocytes reside and the capacity for RVD would provide a useful insight into the volume regulatory response. Fluorescent experiments on isolated cells (thus avoiding the problems of drugs penetrating the cartilage matrix) using inhibitors to the low conductance K^+ channel, various Cl^- channels, actin cytoskeleton and a higher concentration of REV 5901 may further elucidate the mechanism for RVD.

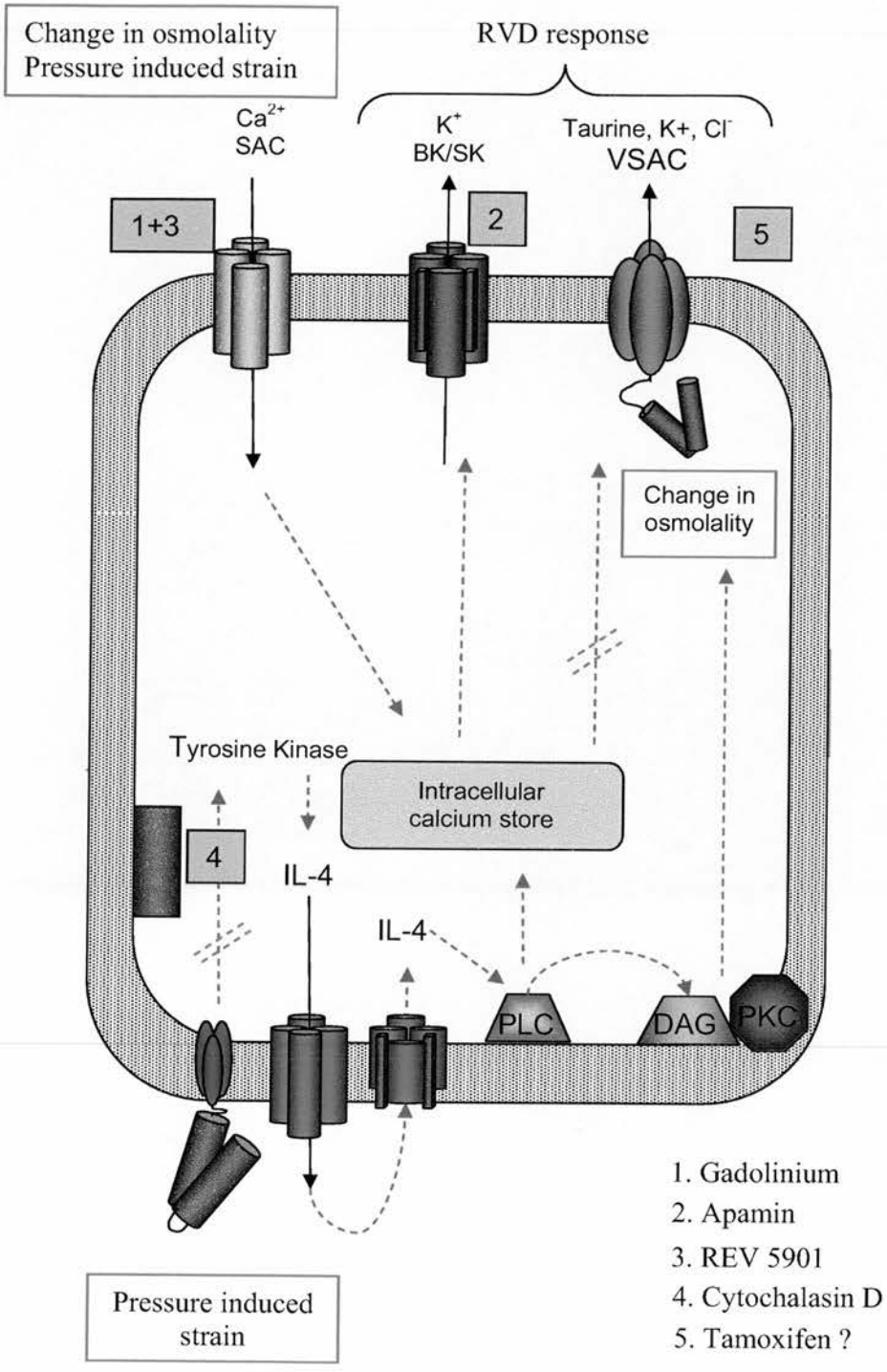

Change in osmolality
Pressure induced strain
RVD response
Ca^{2+}
SAC
K^+
BK/SK
Taurine, K+, Cl^-
VSAC
1+3
2
5
Change in osmolality
Tyrosine Kinase
Intracellular calcium store
4
IL-4
IL-4
PLC
DAG
PKC
Pressure induced strain
1. Gadolinium
2. Apamin
3. REV 5901
4. Cytochalasin D
5. Tamoxifen ?

Figure 6. 7. Proposed mechanism for RVD in human articular chondrocytes.

Based on the data presented in this chapter and the pressure-induced strain response by Milllward-Sadler et al., (1999). A change in osmolality initiates a rise in $[Ca^{2+}]_i$ *mediated by a stretch-sensitive calcium channel that can be inhibited by REV 5901 or* Gd^{3+}*. This may then lead to the activation of the low-conductance apamin-sensitive* K^+ *channel that could be involved in the RVD response. The decrease in osmolality also activates a volume-sensitive, calcium-insensitive anion channel, which is most likely the principal pathway for RVD. A dotted red indicates a pathway not involved in the RVD response. Tamoxifen has been shown as a potential inhibitor of the 'osmolyte channel' as it did appear to block RVD but as the data is preliminary and the drug is highly cytotoxic more work is required.*

Discussion

This study aimed to elucidate some of the mechanism of volume regulation in isolated articular chondrocytes. The role of intracellular calcium (as a potential signalling molecule) was investigated in both the RVD and RVI response, and the requirement for a polymerised F-actin cytoskeleton was studied for RVD. Lastly, the capacity for RVD was investigated in chondrocytes isolated from 'non-degenerate' and 'degenerate' human articular cartilage as a potential explanation for the increase in cell volume associated with increasing pathology.

Volume regulation by freshly-isolated bovine articular chondrocytes

In response to a decrease in extracellular osmolality, ~60% of freshly-isolated chondrocytes had the capacity for volume regulation. RVD was mediated by an 'osmolyte channel' of low specificity that could be inhibited by a pre-incubation with REV 5901 and not by the depolymerisation of the F-actin cytoskeleton. Conversely, a pre-incubation with Gd^{3+} (an inhibitor of stretch-sensitive channels) had no effect on the capacity for RVD and this would suggest that the osmolyte channel is not stretch sensitive. Furthermore, as Gd^{3+} has been shown to inhibit the pressure-induced strain response it would appear that there are two separate pathways, one involved in 'sensing' changes in osmolality and the other in membrane stretch.

The decrease in osmolality initiated a transient rise in $[Ca^{2+}]_i$ in some cells, mediated by an influx from the extracellular environment. Furthermore, as $[Ca^{2+}]_i$ rise was also observed in chondrocytes in an EGTA saline, this would suggest that there was a release from cellular stores. When correlating the RVD response to the rise in $[Ca^{2+}]i$ the data implied that within cartilage there were at least two sub-populations with different

dependencies on calcium. One population (~41%) required a rise in calcium to mediate RVD whereas the other population (~25%) are able to volume regulation without any significant changes in cell calcium. The possibility that within cartilage there are at least two populations is interesting, and may relate to the position of the chondrocytes within the tissue.

Cartilage hydration follows FCD due to the presence of GAG. Simply, the fixed charge density is lowest in the SZ, increase in the MZ and then begins to decrease in the DZ (Maroudas, 1980, 1981). Therefore, physiologically the SZ is better designed at resisting tensile forces whereas the MZ and DZ are better designed to resist static compressive load (Buckwalter & Mankin, 1997a). One could therefore speculate that chondrocytes in the MZ and DZ may behave similarly to changes in osmotic pressure, whereas chondrocytes in the SZ may exhibit a different response.

In is quite possible that a change in osmolality would be more 'dramatic' in the MZ due to the higher water content. Therefore, as changes in extracellular osmolality attenuate matrix synthesis (Urban *et al.*, 1993), that may subsequently compromise the integrity of the ECM, it is conceivable that the more rapid the RVD response, the faster the chondrocytes could adapt. It has previously been shown that there was no significant differences in the rate of RVD between any of the main zones of cartilage (Bush & Hall, 2000), although due to the limits of the equipment used it was not possible to record continuous RVD. Here, using fluorescent microscopy this was possible, and so should there be any differences in the intermediate time points (i.e. Bush & Hall 2001 only studied 1.5, 5, 10, 20mins post RVD) these would be detected. When comparing the

rates of RVD between chondrocytes that did and did not exhibit a rise in $[Ca^{2+}]_i$, there was no significant difference (unpublished observations). Conversely, there was a significant difference in maximal cell swelling. It is therefore conceivable that RVD in chondrocytes is bi-phasic. The first, rapid phase, dependent upon a rise in $[Ca^{2+}]_i$ resulting in a significant amount of RVD over a short time period and the second phase slower and not stimulated by a rise in $[Ca^{2+}]_i$.

Based on this hypothesis, chondrocytes in the MZ and DZ may have a calcium dependent RVD response whereas chondrocytes in the SZ do not (Fig. 7.1). Therefore, as chondrocytes in the SZ may not 'see' a large osmotic challenge in vivo they do not posses the calcium activated RVD response and just have calcium independent RVD. Conversely, MZ and DZ chondrocytes may exhibit more fluctuations in extracellular osmolality and thus in order to retain volume and consequently matrix synthesis, have developed robust, calcium-dependent RVD. Therefore, the removal of extracellular calcium inhibits the activation of this and therefore the apparent decrease in the percentage of cells responding. Interestingly, REV 5901 inhibited ~ 95% of the RVD response (Bush & Hall, 2000; Hall & Kerr, 2000; Bush & Hall, 2001b) and this would therefore imply that despite the mode of activation, RVD may be mediated by the same channel. However, as REV 5901 is not specific for the 'osmolyte channel' it is also possible that two channels reside, similar in structure with different mechanisms of activation. These points all require further study.

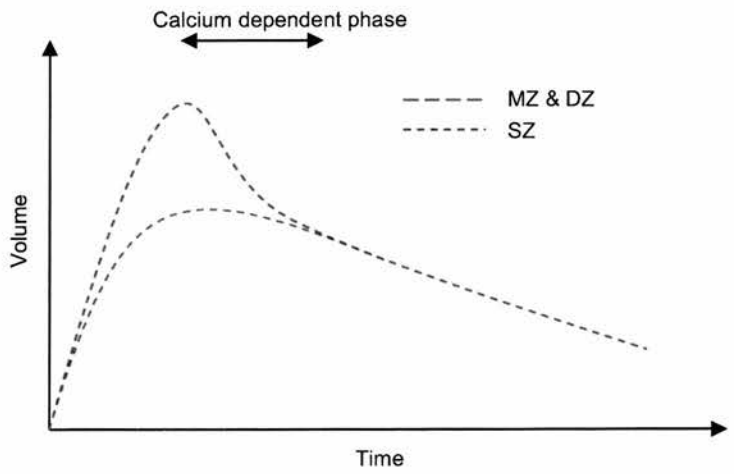


Figure 7. 1. Possible mechanism of RVD in bovine articular chondrocytes.

RVD in bovine articular chondrocytes may be mediated by two separate pathways, one dependent upon $[Ca^{2+}]_i$ *and the other independent. It is possible due to the nature of the cartilage and the forces perceived, that SZ chondrocytes lack the calcium dependent pathway whereas MZ and DZ chondrocytes have a functional calcium-dependent RVD response.*

Here it was shown that isolated bovine articular chondrocytes do not have the capacity for RVI and this may be related to the physical properties of cartilage. During the day, cartilage is slowly compressed (~5% loss in cartilage water per day; (Urban, 1994). Conversely, during horizontal rest, cartilage rehydrates due to the removal of the static compressive load. With this diurnal effect, the capacity for RVI may not actually be required as original volume is essentially always restored. Furthermore, volume regulation (i.e. RVI) may in fact be detrimental to chondrocyte function. An increase in extracellular osmolality increases the polymerisation of the F-actin cytoskeleton in many cell types (Pedersen *et al.*, 1999; Guilak *et al.*, 2002; Lewis *et al.*, 2002) although in chondrocytes it has been shown not to change (Guilak *et al.*, 2002). As F-actin is cortical arranged in *in situ* chondrocytes (Langelier *et al.*, 2000), and is it is involved in maintaining the viscoelastic properties of the cell (Guilak *et al.*, 2002) volume regulation by RVI could infact be detrimental to these properties.

An increase in osmotic pressure results in the decrease of chondrocyte volume although no change in the organization of the F-actin cytoskeleton (Guilak *et al.*, 2002; Erickson *et al.*, 2001; Kerrigan & Hall 2001). Should the chondrocyte respond with RVI this might disrupt the F-actin organization and therefore decrease the chondrocytes resistance to static compressive force. This may then, result in an increase in fragility should the compressive force get to great. The fact that the change in $[Ca^{2+}]_i$ did not correlate with the RVI observed would imply that is it not involved in the volume regulatory response and one could suggest that it has a role in mediating the organization of the cytoskeleton as well as the metabolic activity of the chondrocytes.

Volume regulation in 2D cultured chondrocytes

After a period of three weeks in monolayer culture, there was a significant change in chondrocyte morphology with associated changes in the F-actin organisation. The time in culture had a significant effect on the capacity for volume regulation. In response to a decrease in osmolality, the chondrocytes were still able to volume regulate by a REV 5901-sensitive pathway although had developed a stretch sensitive $[Ca^{2+}]_i$ rise. Furthermore, unlike freshly-isolated chondrocytes 2D cultured cells had the capacity for robust, hyper-osmotic RVI independent of a rise in $[Ca^{2+}]_i$ and also bumetanide insensitive.

It is currently unclear as to why these pathways developed during culture, although they are most likely linked to the phenotype of the cell. For chondrocytes, monolayer culture is far removed from the *in vivo* environment. Chondrocytes do not reside in areas of dense populations, and it has been shown that high-density culture can influence the chondrocyte phenotype (Schulze-Tanzil *et al.*, 2002). Furthermore, upon culture, chondrocytes form strong attachments to the culture plastic most likely mediated in part by the re-organisation of the F-actin cytoskeleton. It is well known that 2D culture results in the loss of the chondrocytic phenotype and the cells become more fibroblastic (Benya & Shaffer, 1981; Benya *et al.*, 1981). It is therefore possible the changes observed in this study are the result of the loss of phenotype.

To quantify these changes, to the chondrocytic phenotype, the experiment would need to be correlated to markers of the differentiated and de-differentiated phenotypes

(collagen II Vs collagen I) and this will indicate whether the changes observed are due to either a change in phenotype or just a change in morphology.

RVD in human articular chondrocytes

As previously mentioned, chondrocytes in OA cartilage have a larger cell volume than those in 'non-degenerate' cartilage (Bush & Hall, 2003). This study has shown that chondrocytes isolated from 'degenerate' cartilage have a functional RVD response and therefore the increase in cell volume may not be attributed to the lack of RVD. As mentioned in chapter 5 (section 5.4.0) other possibilities that may mediated an increase in cell volume would be an activation of the RVI response, an increase in cellular osmolality (due to an up-regulation of protein synthesis) or changes in the extracellular matrix that allow for further swelling. Do date, it is not currently known why chondrocytes have a further increase in volume and this requires further study.

An interesting observation from the human RVD work was that the volume-regulatory response was not inhibited by Gd^{3+} although the rise in $[Ca^{2+}]_i$ was. This (as previously mentioned) has two major implications. The first is that RVD was not mediated by the $\alpha5\beta1$ integrin receptor-ligand complex and secondly RVD was independent of $[Ca^{2+}]_i$. An important consideration when comparing the RVD data here and the pressure induced strain response was that the strain experiments were performed on 2D cultured chondrocytes whereas the RVD experiments were performed on freshly-isolated cells. The authors have shown that the cultured human chondrocytes still expressed the differentiated phenotype (based on collagen expression; (Wright *et al.*, 1996) although

there had been a significant change in morphology. It would therefore be interesting to test the pressure-induced strain response on freshly-isolated chondrocytes and the RVD response on 2D cultured human chondrocytes. If RVD were related to morphology, then one would expect that RVD would be inhibited by Gd^{3+} in 2D cultured chondrocytes therefore suggesting that the pressure-induced strain response is acquired through culture and thus may not reflect the *in situ* environment. Conversely, should RVD remain Gd^{3+} insensitive this would further confirm that pressure induced strain and RVD are mediated by two separate pathways.

Closing remarks

The study of chondrocyte volume regulation in response to changes in extracellular osmolality, may provide an insight in the regulation of matrix synthesis and thus the resilience of the ECM to mechanical forces. Morphological and phenotypic changes as a result of culture may have profound effects on the capacity for volume regulation and care may need to be taken when relating these experiments to *in situ* models. Finally, the increase in cell swelling observed in OA, does not appear to be attributed to the loss of RVD and therefore another explanation remains to be found.

Publications

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Differential mechanisms of intracellular pH (pHi) recovery following acid load to in situ bovine articular chondrocytes within different cartilage zones.

2. Lucas C.D., Kerrigan M.J. and Hall A.C. (2002). J Physiol (Lond) **544P**.
Volume regulation by isolated bovine articular chondrocytes within gels.

3. Kerrigan M.J. and Hall A.C. (2002). J Physiol (Lond) **544P**.
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The role of $[Ca^{2+}]_i$ in mediating regulatory volume decrease in isolated bovine articular chondrocytes.

Appendix 1

Considerations of fluorescent microscopy

Fluorescence microscopy is not without its problems and it is important to address some of each of these and discuss methods in which the potential experimental problems can be avoided. There is always the possibility that fura-2 will become compenmartilised within intracellular compartments including the endoplasmic reticulum and the mitochondria. The sequestering of fura-2 would lead to an osmotic-insensitive background fluorescence that may potentially 'mask' the cytosolic $[Ca^{2+}]_i$ signal and thus interfere with volume measurements. A method often employed to limit compartmentalisation is to decrease the loading temperature to that of room temperature (Roe *et al.*, 1990; Simpson) although there are the problems of attenuating cellular activity at a lower temperature. All the experiments described within this thesis were all performed at 37°C and so fluorophore loading was maintained at this temperature as it was decided (in conjunction with the fact previous studies on chondrocytes have shown 37°C loading to work; (Hall & Bush, 2001) that a decrease in temperature my cause more experimental problems that potential compartmentalisation. An alternative method of direct micro-injection of fura-2 acid would also avoid compenmartilsation but the associated problems of cell damage and decreasing the sample size this was avoided (Simpson, 1997).

Another problem of fluorescent microscopy is that of photobleaching. This is an irreversible chemical reaction in response to strong light where the fluorophore is broken down. Furthermore, although the mechanism are unknown, it has been shown

that dye-loading is also susceptible to light (Simpson, 1997) and would therefore potentially decrease the amount of dye entering the cell. These problems were addressed by always dye-loading the cells in the dark and the system was calibrated when the camera gain was adjusted to give maximal signal with minimal excitation light (data not shown). There is the potential problem where by the fluorophore excitation is low and the camera gain is high and although the images are fine and there is no bleaching, no fluorescent changes are detectable (Kerrigan & Hall; Unpublished observations). Once a suitable excitation light intensity was determined (by adjusted the monochromator slit-widths and the lamp aperture) and the camera gain adjusted these settings were used for all experiments.

Changes in cell volume alter pH_i (Putnam *et al.*, 1998; Gillis *et al.*, 2001) and this may alter the K_d of calcium for fura-2 (Simpson, 1997). These changes could cause problems when looking at fluorescent changes in response to changes in extracellular osmolality leading to either an over or under-estimation. Work by Tsein *et al.*, (Grynkiewicz *et al.*, 1985) have shown that changes in pH_i over the physiological range have very little effect on the spectra for Ca^{2+}-free, Ca^{2+} bound or the Ca^{2+} dissociation constant and therefore this would imply that pH changes will not interfere with the volume measurements made within this thesis.

Another potential problem associated with fura-2 is the products of fura-2 hydrolysis. When fura-2 AM crosses into the cell it is hydrolysed by cellular esterases in to fura-2 and a by-product formaldehyde (Tsien, 1981). This has potential problems, as formaldehyde is cytotoxic and could damage the cell. A study on human red blood and

mast cells have shown that no cytotoxic effects were evident and this was attributed to the high permeability of formaldehyde and thus the avoidance of high concentrations within the cells (Tsien, 1981).

To summarise, fluorescent microscopy is a powerful tool in the study of cell biology and the elucidation of cell function. The associated problems on the most part can be avoided or at least reduced leading the accurate measurement of cellular changes. The ability to measure 'real-time' cellular responses to an extracellular stimulus has, and will continue to be a useful tool in cell biology.

Appendix 2

Preparation of Chondrocyte Membranes

Cells were isolated from pooled full-depth cartilage explants excised from bovine metacarpal and metatarsal pharyngeal joints as previously described (see Materials and Methods) into 380mOsm.kg H_2O^{-1} DMEM. In order to increase the number of cells recovered, the supernatant was filtered using a vacuum filter (through a 50μm mesh) instead of through a cell strainer. After the supernatant was filtered, the cells were washed twice by centrifugation as previously described (see Materials and Methods).

After the second wash, the cells were washed in 190mM NaCl saline by centrifugation (540g; 10mins; 4°C) to remove all traces of culture media. The saline was aspirated, and the pellet re-suspended into 1ml of ice-cold homogenisation buffer (50mM Tris contained in EDTA). The supernatant was then divided into two, transferred into 1ml Eppendorfs and freeze-thawed twice using a dye-ice, iso-propanol bath (~ -90°C). Cells were then sheared using a 1ml syringe and a needle series decreasing in diameter.

To remove large cell debris, the two Eppendorfs were spun (using a bench top centrifuge) at 2000rpm for 15mins (RT°C) and the supernatants removed. The resultant supernatants were subsequently combined and centrifuged at 50,000g for 30mins (4°C). The pellet (containing the cell membranes) was then re-suspended into 50μl of re-suspension buffer (10mM Tris-EDTA pH 6) plus protease inhibitors (in EDTA) and frozen at -20°C until required.

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